

Claim 7 is amended herewith to conform the disorders listed therein to those of Claim 3 so Claim 7 should be allowable if Claim 3 is allowable.

Claim 8 is canceled herewith as being redundant in view of the amendments to Claim 7.

Claims 9 and 10 are canceled herewith to change the dependency from 8 to 7 in view of the cancellation of Claim 8.

Claim 11 has been amended to include limitations of Claim 17 and to correct an error.

Claim 17 has been amended to include the limitations of canceled Claim 6 and to change the dependency in view of cancellation of Claim 6.

A version with markings to show changes made is attached.

We turn now to the rejections.

Claim 6 is rejected under 35 U.S.C. 112, first paragraph. Claim 6 is canceled herewith to reduce the issues. Reconsideration is requested.

Claims 1 and 2 are rejected under 35 U.S.C. 102(a) as being anticipated by Gregory, et al WO 07/297,776. Claims 1 and 2 are canceled herewith to reduce the issues. Reconsideration is required.

Claims 1-6 and 17 were rejected as being obvious over Gregory, et al. U.S. Patent No. 6,172,096 in view of Talley U.S. Patent No. 5,643,933. The advisory action takes the position that Talley teaches COX-2 inhibitors are generally known for treating inflammatory disease and Gregory teaches that COX-2 inhibitors are known for treating liver disease and since chronic hepatitis involves inflammation of the liver, it would be obvious to use COX-2 inhibitors to treat chronic hepatitis.

Reconsideration of the obviousness rejection is requested in view of evidence presented before and new evidence presented herewith and the positions set forth below.

Firstly, the indication in the Advisory Action that COX-2 inhibitors are generally known for treating inflammation needs to be qualified.

COX-2 inhibitors have been shown to be harmful in a colitis model for inflammatory bowel disease and in the case of ulcers. See Reuter, B.K., et al, J. Clin. Invest. 98 No. 9, 2076-2085 (11/96) and Mizuno, H., et al, Gastroenterology 112, 387-397 (1997), copies submitted with the response of May 15, 2002.

Moreover, the Arthritis Advisory Committee of the FDA concluded in 1982 that hepatotoxicity is a class characteristic of NSAIDs. See Zakin, D., et al, Hepatology A Textbook Liver Disease, Volume II, Third Edition, W.B. Saunders, Philadelphia (1996), pages 976 and 977, copy enclosed with the response filed 1/7/02. It is submitted that the Seibert abstract relied on by the PTO to rebut the position that one skilled in the art would apply that conclusion to COX-2 inhibitors, has been overcome by the positions in the response of 5/15/02.

Furthermore, prostaglandins, compounds produced by action of cyclooxygenase-2, have long been known (at least since 1993) to protect against LPS-induced liver injury by downregulating the production of inflammatory cytokines. See Mokuno, Y., et al, Hepatology 30, 1464-1472 (1999), copy enclosed. This indicates administration of a COX-2 inhibitor would deprive a patient of liver protecting prostaglandin.

Thus, the general utility of COX-2 inhibitors to treat inflammatory diseases would be considered to have many exceptions including the treatment of inflammatory liver diseases.

The Advisory Action of 5/29/02 implies that Gregory teaches use of COX-2 inhibitors to treat liver disease. It is submitted that this is overgeneralization. Gregory teaches use of COX-2 inhibitors only for use for treating two liver conditions, namely liver transplant rejection and primary biliary cirrhosis (a disease of unknown cause which may possibly be an autoimmune disorder).

It is submitted that Gregory is suspect even as a teaching for the two liver conditions it mentions. It contains no data in respect to either of these conditions and is indicated to be wrong in respect to application of COX-2 inhibitors to inflammatory bowel disease (column 5, line 63) by Reuter et al discussed above.

Even if Gregory is given credit for a teaching that would be accepted by one skilled in the art, as teaching the usefulness of COX-2 inhibitors to treat or prevent liver transplant rejection and to treat primary biliary cirrhosis, it cannot be taken as refuting the 1982 conclusion of the Arthritis Advisory Committee that suggests that COX-2 inhibitors are contraindicated in the case of liver diseases. The 1982 conclusion is still in effect. Medical doctors still do not prescribe COX-2 inhibitors in the case of liver disorders. The 2001 PDR indicates that COX-2 inhibitors should not be utilized in the case of liver disorders. See copies of pages 2051 and 2484 of the 2001 PDR, copy of relevant portions attached. Thus, Gregory has not been accorded the weight attributed to it in the office action, by those skilled in the art even though WO 97/29776, applied in the office action, was published in 1997.

The advisory action seems to take the position that all inflammatory liver disorders including transplant rejection and primary biliary cirrhosis (Gregory) and hepatitis (the claims) are analogous for treatment purposes. However, no evidence has been submitted showing this.

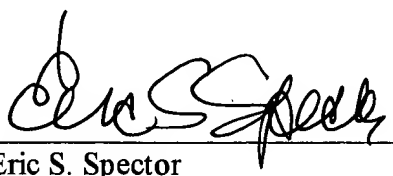
Moreover, there is contrary evidence as of May/June 1997. In this regard, see Anderson, F. H., et al, Can. J. Gastroenterol. 11, No. 4, 294-297 (May/June 1997), copy enclosed, which presents data that the NSAID ketoprofen in use in a combination of ketoprofen and interferon does not improve interferon-resistant hepatitis C. Moreover, this finding is confirmed by Zanski, J.-P, et al, Hepatology 27, 862-867 (1998), copy enclosed, which presents data showing the NSAID tenoxicam is unable to increase response rate in patients with chronic hepatitis C treated by alpha interferon.

Thus, evidence suggests there is no analogy between the inflammatory liver disorders disclosed in Gregory and the inflammatory liver disorders as claimed, so far as treatment is concerned, even if Gregory is given credit for one skilled in the art accepting that it teaches useful treatment of liver transplant rejection and primary biliary cirrhosis (which applicant contends should not be the case).

Allowance is requested.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

Claims 1, 2, 6 and 8 are canceled.

Claim 7 is amended as follows:

7. (Amended) A method of treating a patient with a virus-caused liver disease selected from the group consisting of chronic viral hepatitis B and chronic viral hepatitis C comprising administering to said patient a cyclooxygenase-2 inhibiting amount of selective inhibitor of cyclooxygenase-2 and therapeutic amount(s) of anti-viral drug(s).

Claim 9 is amended as follows:

9. (Amended) The method of Claim 7[8], wherein the selective inhibitor of cyclooxygenase-2 is 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide.

Claim 10 is amended as follows:

10. (Amended) The method of Claim 7[8] wherein the selective inhibitor of cyclooxygenase-2 is 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide.

Claim 11 is amended as follows:

11. (Amended) The method of Claim 7[8] wherein the selective inhibitor of cyclooxygenase-2 directly inhibits the enzyme cyclooxygenase-2 and also inhibits the synthesis of cyclooxygenase-2 protein and contains phenyl group with two or more substituents selected from the group consisting of hydroxy and C<sub>1-4</sub>-alkoxy on the phenyl group.

Claim 17 is amended as follows:

17. (Amended) The method of Claim 3[6] wherein the selective inhibitor of cyclooxygenase-2 directly inhibits the enzyme cyclooxygenase-2 and also inhibits the synthesis of cyclooxygenase-2 protein and contains phenyl group with two or more substituents selected from the group consisting of hydroxy and C<sub>1-4</sub>-alkoxy on the phenyl group.

# Prostaglandin E<sub>1</sub> Protects Against Liver Injury Induced by *Escherichia coli* Infection via a Dominant Th2-Like Response of Liver T Cells in Mice

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Prostaglandin E series (PGEs) are known to protect against lipopolysaccharide (LPS)-induced liver injury by down-regulating the production of inflammatory cytokines. We show here a novel mechanism whereby prostaglandin E<sub>1</sub> protects mice against liver injury after *Escherichia coli* infection. Prostaglandin E<sub>1</sub> administration suppressed circulating interleukin 12 (IL-12) levels but increased the IL-10 production after *E. coli* challenge. Furthermore, prostaglandin E<sub>1</sub>- $\alpha$ -cyclodextrin (PGE<sub>1</sub>- $\alpha$ -CD) shifted the Th1/Th2 balance of CD3<sup>int</sup> IL-2R $\beta$ <sup>+</sup> T cells in the liver to a dominant Th2-like response. Neutralization of endogenous IL-4 by administration of anti-IL-4 monoclonal antibody (mAb) diminished the inhibitory effect of prostaglandin E<sub>1</sub> on liver injury after *E. coli* challenge. These results suggested that the Th2-like response of liver T cells may be at least partly involved in the mechanism whereby prostaglandin E<sub>1</sub> protects against *E. coli*-induced liver injury. (HEPATOLOGY 1999; 30:1464-1472.)

Prostaglandins, oxygenated polyunsaturated fatty acids containing a cyclopentane ring structure, are produced by the action of the enzyme cyclooxygenase on arachidonic acid liberated from membrane phospholipids.<sup>1</sup> Macrophages, follicular dendritic cells, and fibroblasts synthesize prostaglandin E series (PGEs) as major products of arachidonic acid metabolism.<sup>2-4</sup> PGEs can be induced by a plethora of membrane-perturbing stimuli, including interleukin 1 (IL-1),

tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), the cross-linking of Fc receptor, lipopolysaccharide (LPS), and components of the complement cascade.<sup>1,5</sup> Effects of PGEs result from their binding to PGE receptor subtypes, EP<sub>2</sub> and EP<sub>4</sub>, that then stimulate production of the second messenger cyclic-3',5' adenosine monophosphate (cAMP).<sup>6-8</sup> Increased levels of cAMP are known to inhibit activation of nuclear factor  $\kappa$ B via retarded degradation of inhibitory factor  $\kappa$ B, whereas they stimulate activating transcription factor/cAMP response element (ATF/CRE) site-mediated gene transcription.<sup>9-12</sup> The production of some cytokines, the genes of which contain a nuclear factor  $\kappa$ B site in the 5' regulatory region, such as TNF- $\alpha$  and IL-12, is inhibited by cAMP elevating agents, whereas the expression of cytokine genes containing an activating transcription factor/cAMP response element-1-like site but not a nuclear factor  $\kappa$ B site in the 5' regulatory region, such as IL-10, is up-regulated by the cAMP-dependent pathway.<sup>12-14</sup> It has been shown that PGEs increase LPS-induced IL-10 production by peritoneal macrophages<sup>15</sup> and inhibit IL-12 production in an IL-10-independent manner.<sup>13</sup> Furthermore, PGEs, as well as other agents that increase cAMP, profoundly decrease the production of IL-2 and interferon gamma by the Th1 subset.<sup>1,16</sup> These findings suggest that PGEs can be used as anti-inflammatory agents in patients with excessive inflammatory responses.

The incidence of infection with Gram-negative bacteria such as *Escherichia coli* has increased in recent years among patients undergoing abdominal surgery. These infections frequently result in liver injury and fatal shock, which are caused by endotoxin/LPS derived from Gram-negative bacteria. Several cytokines are involved in LPS-induced liver injury. Massive TNF- $\alpha$  release from macrophages plays a central role in LPS-induced liver injury.<sup>17-19</sup> IL-12 also plays an important role in LPS-induced liver injury via stimulation of natural killer (NK) cells to produce interferon gamma,<sup>20</sup> which potentiates the TNF- $\alpha$ -induced, Fas/Fas ligand-mediated and perforin-mediated apoptosis in hepatocytes.<sup>21,22</sup> The liver contains unique T cells expressing intermediate intensity of T cell receptor and IL-2R $\beta$ , which are now known to correspond to NK T cells in regard to their restricted TCR  $\alpha\beta$  repertoire and restriction of major histocompatibility complex (MHC) class I-like Ag.<sup>23-26</sup> NK T cells in the liver have recently been found to be an essential target of IL-12 for NK-like effector mechanisms such as interferon gamma production and cytotoxicity.<sup>23,25</sup> Therefore, it is possible that NK T cells in the liver may contribute to LPS-induced liver damage via NK-like effector mechanisms.

Abbreviations: PGEs, prostaglandin E series; IL-1, interleukin 1; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; LPS, lipopolysaccharide; cAMP, cyclic-3', 5' adenosine monophosphate; NK, natural killer; TCR, T-cell receptor; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>- $\alpha$ -cyclodextrin; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>- $\alpha$ -cyclodextrin;  $\alpha$ -CD,  $\alpha$ -cyclodextrin; Ig, immunoglobulin; IP, intraperitoneal; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; CD3<sup>int</sup>, intermediate intensity of CD3; PCR, polymerase chain reaction; mRNA, messenger RNA.

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We previously reported that prostaglandin  $E_2$ - $\alpha$ -cyclodextrin (PGE<sub>2</sub>) protects against liver injury after *E. coli* infection although it hampers the resolution of the infection in mice.<sup>27</sup> In the present study, we focused on the effect of prostaglandin  $E_1$ - $\alpha$ -cyclodextrin (PGE<sub>1</sub>) on liver T cells in protection against liver injury induced by *E. coli* infection. Our results showed that PGE<sub>1</sub> administration shifted the Th1/Th2 balance of liver T cells to a Th2-like response and that neutralization of endogenous IL-4 by anti-IL-4 monoclonal antibody (mAb) exaggerated the *E. coli*-induced liver damage. The implications of these findings for the mechanisms whereby PGE<sub>1</sub> protects against liver injury induced by *E. coli* infection are discussed.

## MATERIALS AND METHODS

**Animals and Microorganisms.** C3H/HeN mice were purchased from Japan SLC (Shizuoka, Japan). These mice were bred in our institute under specific pathogen-free conditions. Eight- to 10-week-old female mice were used for the experiments. All experiments were done along the criteria outline in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science. *E. coli* (American Type Culture Collection No. 26; Manassas, VA) grown in brain-heart infusion broth (Difco Laboratories, Detroit, MI) was washed repeatedly, resuspended in phosphate-buffered saline (PBS), and stored at  $-70^\circ\text{C}$  in small aliquots until use. The concentration of bacteria was quantitated by plate counts.

**Abs and Reagents.** Biotin-conjugated anti-CD3 $\epsilon$  mAb, fluorescein isothiocyanate-conjugated anti-TCR  $\alpha\beta$  mAb, purified rat anti-mouse IL-2R $\beta$  mAb, and phycoerythrin-conjugated anti-rat immunoglobulin G (IgG) mAb were purchased from PharMingen (San Diego, CA). Red-613-conjugated streptavidin was purchased from Life Technologies (Gaithersburg, MD). PGE<sub>1</sub> and  $\alpha$ -cyclodextrin ( $\alpha$ -CD) were provided by Ono Chemical (Osaka, Japan). PGE<sub>1</sub> includes 2.88% prostaglandin  $E_1$ . Anti-mouse CD3 $\epsilon$  mAbs (135-2C11), anti-mouse IL-4 mAb (rat IgG1, 11B11), anti-mouse IL-10 mAb (SXC-1), and isotype control rat IgM mAb (WFL4F12.3) were obtained by growing hybridoma cells in a serum-free medium (medium 101; Nissui Pharmaceutical, Tokyo, Japan) and collecting the supernatant. Abs were then concentrated and purified by 50% ammonium sulfate precipitation. The purity of the preparation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the concentration of Ab was determined by the Lowry method. The mAbs, diluted to 1 mg/mL in PBS, were stored at  $-70^\circ\text{C}$  until use. Isotype control rat IgG1 Ab was obtained from Inter-Cell Technologies (Hopewell, NJ).

**Treatment of Mice.** Mice were injected with PGE<sub>1</sub> or with the same dose of  $\alpha$ -CD (as the control) dissolved in 0.5 mL of PBS. Three hours after the PGE<sub>1</sub> or  $\alpha$ -CD challenge, mice were inoculated with *E. coli* at a dose of  $1.0 \times 10^8$  colony-forming unit (CFU)/mouse ( $1/5$  LD<sub>50</sub>) in 0.5 mL of PBS. After being assessed for serum transaminase activity, mice were inoculated with *E. coli* at a dose of  $1.0 \times 10^9$  CFU/mouse ( $2 \times$  LD<sub>50</sub>). All injections were performed via intraperitoneal (IP) administration. Blood was obtained by a retro-orbital plexus puncture at 3, 8, and 12 hours after *E. coli* challenge, and serum levels of TNF- $\alpha$ , interferon gamma, IL-4, IL-10, and IL-12 p40 were determined at these time points, respectively.

**Assay for Serum Alanine Aminotransaminase Activity.** Liver injury was assayed by serum alanine aminotransaminase (ALT) activity. This activity was determined using the serum transaminase test kit (DIA-latron, Tokyo, Japan). Briefly, 40  $\mu\text{L}$  of the serum sample was incubated with 200  $\mu\text{L}$  of L-alanine and -ketoglutaric acid solution, respectively, for 30 minutes at  $37^\circ\text{C}$  for ALT. Twenty minutes after the addition of 200  $\mu\text{L}$  of 2,4-dinitrophenylhydrazine, 2 mL of 0.4 N NaOH was added and by visible light absorption was measured at 505 nm. ALT activities (IU/L) were calculated from the standard curve.

**Bacterial Growth in Omentum.** After infection, peritoneal exudates were obtained from the peritoneal cavity by lavage with 3 mL of Hank's balanced salt solution. Serial dilutions of the exudate samples were plated to determine the number of viable bacteria. For enumeration of counts of viable bacteria in the liver, the liver was perfused with 8 mL of sterile Hank's balanced salt solution to wash out bacteria in the blood vessels immediately after mice were bled. The livers and spleen were removed and separated into sterile Teflon-coated homogenizers (Asahi Techno Glass Co., Tokyo, Japan) containing 5 mL of cold PBS. After each organ was homogenized thoroughly, the bacterial counts in the homogenates were established by plating serial 10-fold dilutions in sterile distilled water on tryptic soy agar (Nissui Laboratories, Detroit, MI). Colonies were counted 24 hours later, after incubation at  $37^\circ\text{C}$ .

**Histological Studies.** Livers were removed from control or PGE<sub>1</sub>-treated mice 8 hours after infection with *E. coli* at a dose of  $1.0 \times 10^9$  CFU/mouse. The livers were fixed with 10% buffered formalin, paraffin-embedded, and stained with hematoxylin-eosin for light microscopic examination.

**Preparation of Liver Lymphocytes.** Mice were infected IP with *E. coli* at a dose of  $1/5$  LD<sub>50</sub> ( $1.0 \times 10^8$  CFU/mouse) in 0.5 mL of PBS on day 0. Fresh livers were immediately perfused with sterile Hank's balanced salt solution through the portal vein to wash out all remaining peripheral blood and then meshed with stainless steel mesh. After the coarse pieces were removed by centrifugation at 50g for 1 minute, the cell suspensions were again centrifuged, resuspended in 8 mL of 45% Percoll (Sigma Chemical Co., St. Louis, MO), and layered on 5 mL of 67.5% Percoll. The gradients were centrifuged at 600g at  $20^\circ\text{C}$  for 20 minutes. Lymphocytes at the interface were harvested and washed twice with Hank's balanced salt solution.

**Flow Cytometry Analysis.** For 3-color analysis, liver lymphocytes were incubated with saturating amounts of biotin-conjugated and purified Abs for 30 minutes at  $4^\circ\text{C}$ . Cells were washed twice and incubated with fluorescein isothiocyanate-, phycoerythrin-, and Red-613-conjugated Abs for 30 minutes. Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). We carefully gated cells by forward and side light scattering for the live lymphocytes. The data were analyzed using FACSCalibur research software (Becton Dickinson).

**Cytokine Assays.** TNF- $\alpha$ , interferon gamma, IL-4, IL-10, and IL-12 levels in serum were determined by enzyme-linked immunosorbent assay (ELISA). ELISAs for TNF- $\alpha$  and IL-10 were performed in triplicate using PharMingen mAbs according to the manufacturer's instructions. ELISAs for interferon gamma, IL-4, and IL-12 were performed using Genzyme mAbs according to the manufacturer's instructions (Genzyme, Cambridge, MA).

**Sorting of CD3<sup>int</sup> IL-2R $\beta$ <sup>+</sup> and CD3<sup>+</sup> IL-2R $\beta$ <sup>-</sup> T Cells.** The intermediate intensity of CD3 (CD3<sup>int</sup>) IL-2R $\beta$ <sup>+</sup> T cells and CD3<sup>+</sup> IL-2R $\beta$ <sup>-</sup> T cells were purified by cell sorting using a FACSVantage (Becton Dickinson) electric cell sorter from the harvested liver lymphocytes 24 hours after *E. coli* infection. The purity of sorted cells were more than 99% (data not shown).

**Expression of Interferon Gamma and IL-4 Genes.** Total RNA was extracted by the acid guanidium-phenol-chloroform method from IL-2R $\beta$ <sup>+</sup> and IL-2R $\beta$ <sup>-</sup> T cells purified by cell sorting. Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) were performed using a complementary DNA cycle kit (Invitrogen Corp., San Diego, CA). Serial dilutions of total RNA were primed with 20 pmol of a random primer in 20- $\mu\text{L}$  reaction mixtures for reverse transcription. Synthesized complementary DNA were amplified by PCR using primers derived from the murine complementary DNA. The PCR was performed on a PCR thermal cycler (Takara Corp., Tokyo, Japan). PCR cycles were run for 30 seconds at  $94^\circ\text{C}$ , 30 seconds at  $54^\circ\text{C}$ , and 30 seconds at  $72^\circ\text{C}$ . Before the first cycle, a denaturation step for 7 minutes at  $94^\circ\text{C}$  was included, and after 25 cycles the extension was prolonged for 4 minutes at  $72^\circ\text{C}$ . The specific primers were as follows: IL-4 sense, 5'-CGAAGAACACCA-CAGAGAGTGAGCT-3'; IL-4 antisense, 5'-GACTCAT TCATGGTG-CAGCTTATCG-3'; interferon gamma sense, 5'-AGCGGCTGACT-



GAAGTCAGATTGTAG-3'; and interferon gamma antisense, 5'-GTCACAGTTTTCAGCTGTATAGGG-3'. The PCR product was subjected to electrophoresis on a 1.5% agarose gel (Life Technologies) and transferred to a GeneScreen Plus filter (NEN Life Science Products, Boston, MA), and probes were labeled with [ $\gamma$ - $^{32}$ P]adenosine triphosphate by using a Megalabel 5'-labeling kit (Takara Shuzo Co. Ltd., Otsu, Japan) according to the manufacturer's instructions. Oligonucleotide probes were as follows: IL-4, 5'-GAGTCTCTG-CAGCTCCATGA-3'; and interferon gamma, 5'-GGTCACTG-CAGCTCTGAATG-3'. For hybridization, the filters were incubated in 1 mol/L NaCl, 1% sodium dodecyl sulfate, 10% dextran sulfate, 100  $\mu$ g of heat-denatured salmon sperm DNA per mL for 18 hours at 60°C, and then the filters were washed in 2  $\times$  standard saline citrate, 1% sodium dodecyl sulfate for 15 minutes at 60°C. The radioactivity of each band of PCR product was analyzed using a Fujix BAS2000 bio-image analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

**Interferon Gamma and IL-4 Assay.** Liver lymphocytes were obtained by the same method previously described. Tissue culture 96-well plates were incubated overnight at 4°C with 50  $\mu$ g/mL anti-CD3e mAb (135-2C11). The plates were then washed thoroughly. The harvested lymphocytes ( $5 \times 10^5$ /well) were incubated in the anti-CD3e mAb-coated plates for 48 hours. Interferon gamma and IL-4 levels in the culture supernatants were determined by ELISA (Genzyme).

**Statistical Analysis.** Data were analyzed by Student's *t* test, and a Bonferroni correction was applied for multiple comparison. The value of *P* < .05 was considered statistically significant.

## RESULTS

**Effects of PGE<sub>1</sub> on Liver Injury and Bacterial Growth After *E. coli* Infection.** A lethal dose of *E. coli* ( $1 \times 10^9$  CFU/mouse) was injected IP into mice 3 hours after administration of 87.5 mg/kg of PGE<sub>1</sub> or  $\alpha$ -CD. PGE<sub>1</sub> treatment significantly decreased serum ALT levels compared with control  $\alpha$ -CD at 8 hours and 12 hours after *E. coli* infection (Fig. 1A, *P* < .05). Dose responses of PGE<sub>1</sub> for protection against liver injury after infection are shown in Fig. 1B. Administration of more than 17.5 mg/kg PGE<sub>1</sub> (0.5 mg/kg as prostaglandin E<sub>1</sub>) significantly protected against *E. coli*-induced liver injury as assessed by the serum ALT level 8 hours after infection. We used 87.5 mg/kg of PGE<sub>1</sub> (2.5 mg/kg as prostaglandin E<sub>1</sub>) as the optimal dose for protection against liver injury in the subsequent experiments. The maximal effect was obtained when PGE<sub>1</sub> was administered 3 hours before the *E. coli* challenge (data not shown).

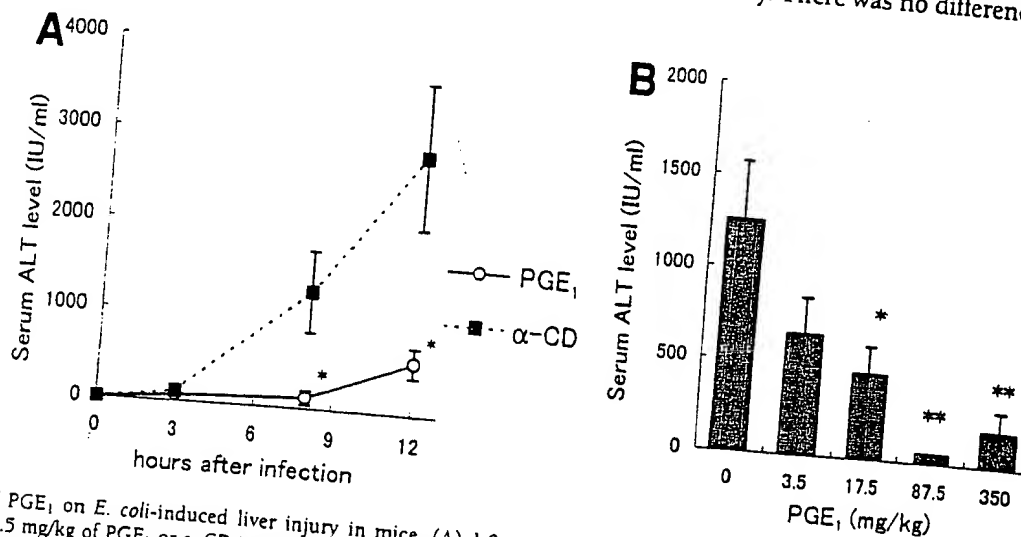


FIG. 1. Effect of PGE<sub>1</sub> on *E. coli*-induced liver injury in mice. (A)  $1.0 \times 10^9$  CFU/mouse of *E. coli* ( $2 \times LD_{50}$ ) was injected IP 3 hours after the administration of 87.5 mg/kg of PGE<sub>1</sub> or  $\alpha$ -CD in PBS. Sera were collected from mice after *E. coli* challenge and measured for ALT activities. (B) Mice were injected IP with indicated doses of PGE<sub>1</sub> before being injected with  $1.0 \times 10^9$  CFU/mouse of *E. coli*. Data are representative of 3 separate experiments expressed as the mean  $\pm$  SD for 5 mice in an experiment. \**P* < .05 versus the control group. \*\**P* < .01 versus the control group.

Consistent with serum ALT activities, pretreatment with PGE<sub>1</sub> reduced histological changes caused by challenge with a lethal dose of *E. coli* including: marked infiltration of inflammatory cells in the liver; massive necrosis of hepatocytes, characterized by cell swelling and focal change; hepatocyte apoptosis showing cell shrinkage and chromatin condensation; and hemorrhage (Fig. 2).

Liver injury by infection with Gram-negative bacteria is caused mainly by LPS derived from bacteria. Therefore, we studied if PGE<sub>1</sub> had an effect on the levels of LPS released from *E. coli* through inhibition of bacterial growth and in turn suppress the liver injury. However, the number of bacteria in the liver were the same as that in the control mice 12 hours and 24 hours after *E. coli* infection (Fig. 3). These results indicate that suppression of liver injury by PGE<sub>1</sub> is not caused by the difference in bacterial loads. On the other hand, the PGE<sub>1</sub> treatment delayed the clearance of bacteria at 36 hours and 60 hours after *E. coli* infection (Fig. 3), suggesting that host defense mechanisms are impaired by exogenous PGE<sub>1</sub>.

**Effects of PGE<sub>1</sub> on the Serum Cytokine Levels After *E. coli* Challenge.** cAMP-increasing agents such as PGE<sub>2</sub> are known to inhibit TNF- $\alpha$  and IL-12 production, but enhance IL-10 synthesis by macrophages in response to LPS stimulation.<sup>12,15,27,28</sup> As shown in Fig. 4, serum TNF- $\alpha$  and IL-12 levels were maximal 3 hours after *E. coli* infection, whereas the IL-10 level reached a peak 8 hours after infection in both control and PGE<sub>1</sub>-treated mice. IL-12 levels were significantly suppressed by *in vivo* administration of PGE<sub>1</sub> (*P* < .05), but there was no difference in TNF- $\alpha$  production between control and PGE<sub>1</sub>-treated mice. On the other hand, levels of IL-10 were significantly higher at 3 hours and 8 hours after *E. coli* infection in PGE<sub>1</sub>-treated mice than in control mice (*P* < .05). Serum IL-4 or interferon gamma were not detected in either control mice or PGE<sub>1</sub>-treated mice at any stage after *E. coli* infection (data not shown).

**Effect of PGE<sub>1</sub> on Liver T Cells After *E. coli* Infection.** NK and T cells in the liver are thought to be responsible for liver injury in several models of hepatic failure associated with endotoxin.<sup>20-22</sup> We next examined the effect of PGE<sub>1</sub> on the relative numbers of NK and T cells in the liver after *E. coli* inoculation by flow cytometry. There was no difference in the numbers of

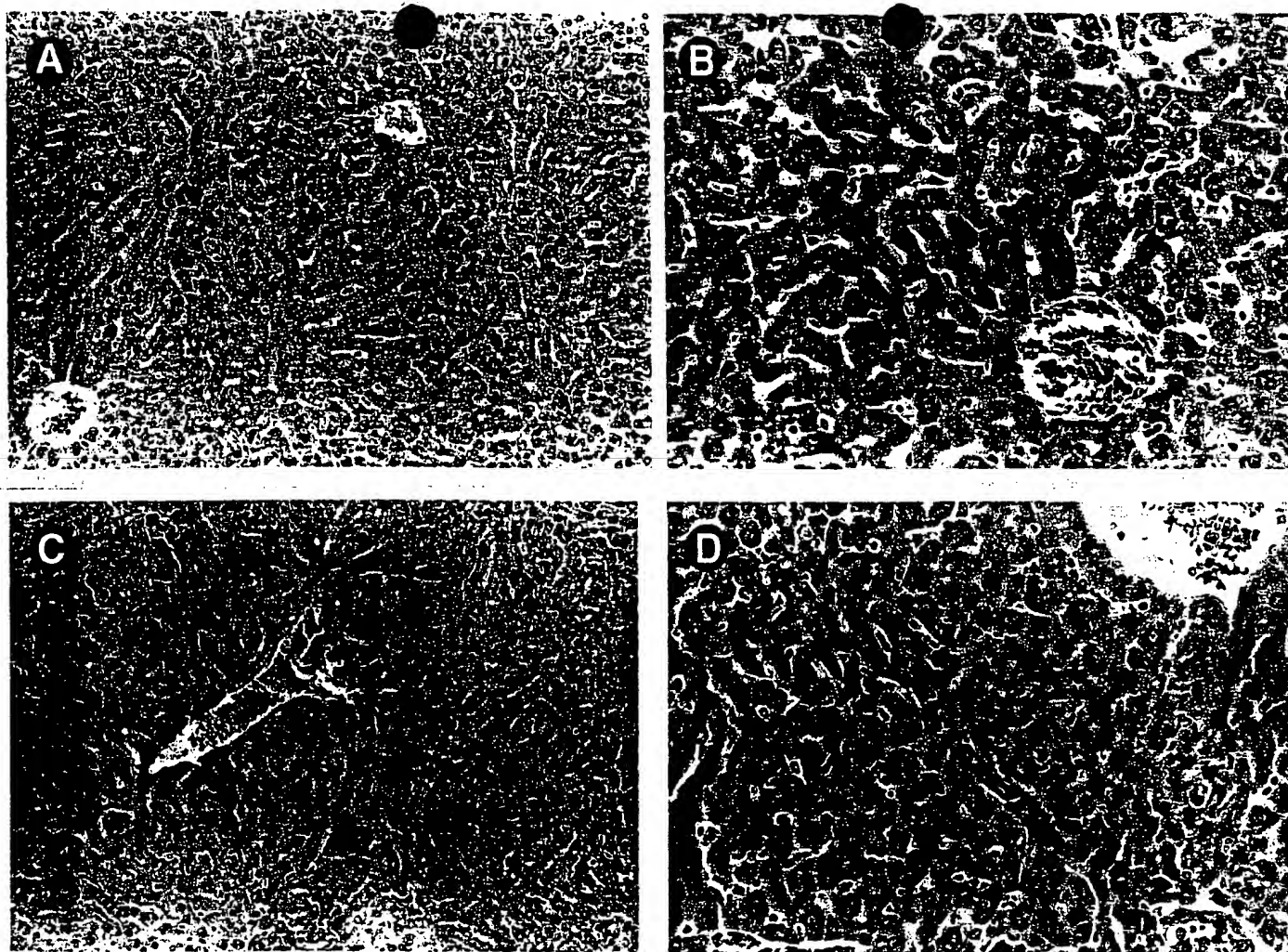


FIG. 2. Effect of  $\text{PGE}_1$  on light microscopic changes in livers from mice infected with *E. coli*. The livers were obtained 8 hours after inoculation of  $1 \times 10^9$  *E. coli* from  $\text{PGE}_1$ -treated mice or  $\alpha$ -CD-treated mice. (A and B) Liver specimens from  $\text{PGE}_1$ -treated mice. (C and D) Liver specimens from  $\alpha$ -CD-treated mice. (Original magnification [A and C]  $\times 133$ ; [B and D]  $\times 400$ .)

$\text{CD3}^- \text{IL-2R}\beta^+$  cells, corresponding to NK cells, or  $\gamma\delta$  T cells in the liver between control and  $\text{PGE}_1$ -treated mice at any stage after *E. coli* infection. On the other hand,  $\text{CD3}^{\text{int}} \text{IL-2R}\beta^+$  cells, which correspond to NK T cells,<sup>23-26</sup> appeared

to decrease in number in  $\text{PGE}_1$ -treated mice compared with control mice at 24 hours after *E. coli* infection (Table 1).

To determine the qualitative difference in liver T cells between control mice and  $\text{PGE}_1$ -treated mice, we sorted T cells bearing  $\text{CD3}^{\text{int}} \text{IL-2R}\beta^+$  and  $\text{CD3}^+ \text{IL-2R}\beta^-$  from the liver mononuclear cells of mice infected with *E. coli* 24 hours previously and examined the expression of messenger RNAs (mRNAs) specific for IL-4 and interferon gamma by means of cytokine reverse transcription-PCR. As shown in Fig. 5A, the expression level of IL-4 mRNA in freshly isolated  $\text{CD3}^{\text{int}} \text{IL-2R}\beta^+$  T cells from  $\text{PGE}_1$ -treated mice was much higher than that from control mice, whereas interferon gamma mRNA was expressed abundantly by the T cells in control mice (Fig. 5A). The expression of IL-4 and interferon gamma mRNA in freshly isolated  $\text{CD3}^+ \text{IL-2R}\beta^-$  T cells remained at low levels in both  $\text{PGE}_1$ -treated mice and control mice.

We next examined cytokine production by liver T cells from *E. coli*-infected mice in response to immobilized anti-CD3e mAb. As shown in Fig. 5B, interferon gamma production by liver T cells from  $\text{PGE}_1$ -treated mice was significantly decreased compared with that by liver T cells from control mice 24 hours after *E. coli* infection ( $P < .05$ ). In contrast, IL-4 production by liver T cells was significantly increased in  $\text{PGE}_1$ -treated mice compared with that in control mice ( $P <$

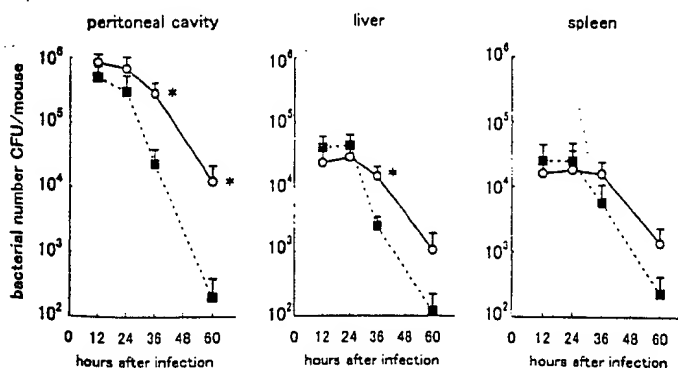


FIG. 3. Bacterial growth in the peritoneal cavity, liver, and spleen of mice infected IP with  $1.0 \times 10^8$  CFU/mouse of *E. coli* 3 hours after inoculation of 0.5 mL of  $\text{PGE}_1$  (87.5 mg/kg) in PBS, or the same amount of  $\alpha$ -CD in PBS. Data are representative of 3 separate experiments and are expressed as the mean  $\pm$  SD for 5 mice in an experiment. \* $P < .05$  versus the control group. (○),  $\text{PGE}_1$ ; (■),  $\alpha$ -CD.

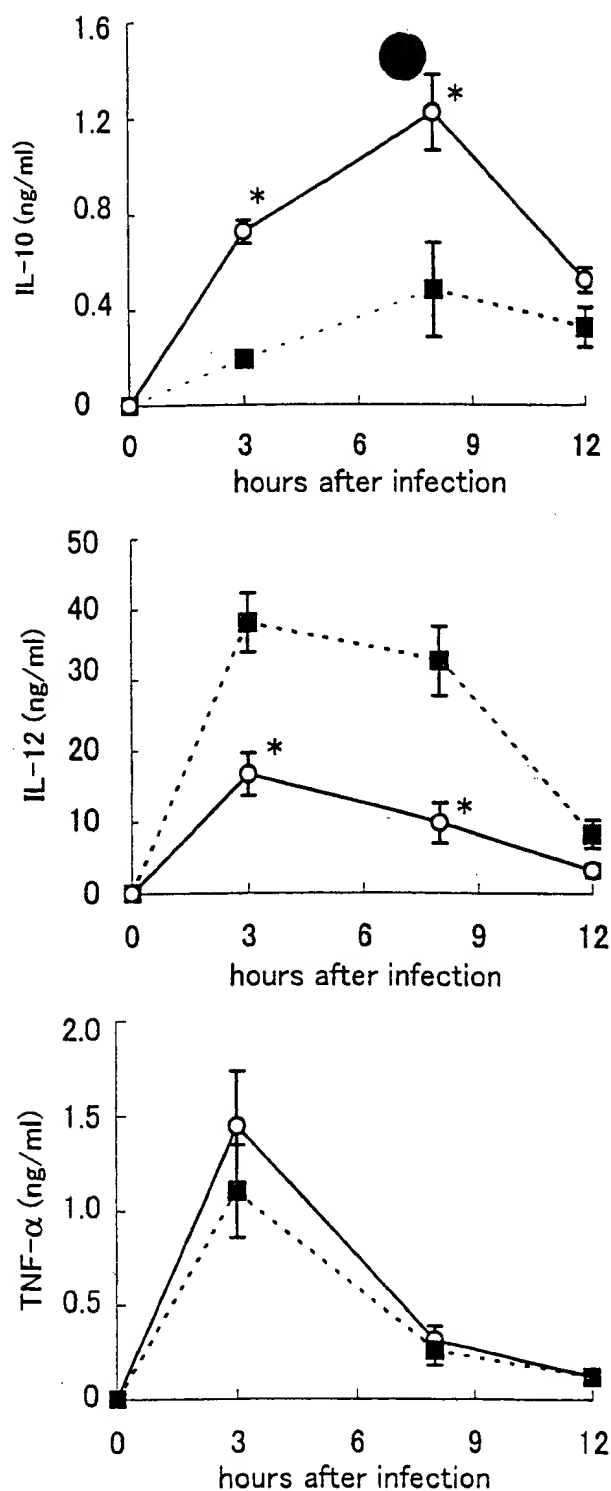


FIG. 4. In vivo effect of PGE<sub>1</sub> on TNF-α, IL-10, and IL-12 in serum of mice infected with *E. coli*. PGE<sub>1</sub> at a dose of 87.5 mg/kg or the same amount of α-CD was injected IP 3 hours before  $1.0 \times 10^8$  CFU of *E. coli* challenge. TNF-α, IL-10, and IL-12 levels in the serum were determined by ELISA. Data are representative of 3 separate experiments and are expressed as the mean  $\pm$  SD for 5 mice in an experiment. \* $P < .05$  versus the control group. (○), PGE<sub>1</sub>; (■), α-CD.

.05). These results suggested that the CD3<sup>int</sup> IL-2Rβ<sup>+</sup> T cells in the liver of PGE<sub>1</sub>-treated mice showed a predominant Th2-like response after the *E. coli* infection.

**Effect of Anti-IL-4 mAb or Anti-IL-10 mAb on Liver Injury After *E. coli* Infection.** We found that liver CD3<sup>int</sup> IL-2Rβ<sup>+</sup> T cells were shifted to Th2 type after PGE<sub>1</sub> treatment in mice

infected with *E. coli*. To determine whether the dominant Th2-like response of liver T cells is responsible for the inhibitory effect of PGE<sub>1</sub> on liver injury after *E. coli* infection, mice were injected IP with anti-IL-4 neutralizing mAb (11B11) at 3 hours before the *E. coli* challenge, and serum ALT levels were examined. As shown in Fig. 6A, administration of anti-IL-4 mAb diminished the inhibitory effect of PGE<sub>1</sub> on *E. coli*-induced liver injury. We also found that administration of PGE<sub>1</sub> enhanced IL-10 release in the serum after *E. coli* challenge. To further investigate whether the increased IL-10 production contributed to the protection against *E. coli*-induced liver injury by PGE<sub>1</sub>, mice were injected IP with anti-IL-10 mAb (SXC-1) 3 hours before the *E. coli* challenge. As shown in Fig. 6B, the anti-IL-10 mAb treatment decreased the effect of PGE<sub>1</sub> on the liver injury. Taken together, these results suggested that both IL-4 and IL-10 are responsible for the protection against liver injury by PGE<sub>1</sub>.

## DISCUSSION

cAMP-increasing agents such as prostaglandin E series are known to protect against LPS-induced liver injury by down-regulating the production of inflammatory cytokines.<sup>27,29,30</sup> Here, we show a novel mechanism whereby PGE<sub>1</sub> suppresses liver injury after *E. coli* infection. PGE<sub>1</sub> treatment shifted the Th1/Th2-like balance in liver T cells to a dominant Th2-like response, and neutralization of endogenous IL-4 by anti-IL-4 mAb administration significantly decreased the suppressive effect of PGE<sub>1</sub> on the liver injury. These results suggested that a dominant Th2-like response of liver T cells is at least partly responsible for PGE<sub>1</sub>-induced suppression of liver injury after *E. coli* infection.

Endotoxin/LPS derived from gram-negative bacteria, such as *E. coli*, play an important role in the pathogenesis of liver injury associated with sepsis. LPS-induced liver injury is known to be mediated by excessive amounts of TNF-α and interferon gamma production, which induce apoptosis of hepatocytes.<sup>22,31</sup> IL-12 is known to be a potent stimulator of interferon gamma production by NK and T cells,<sup>20,32-35</sup> whereas IL-10 shuts down interferon gamma synthesis by these cells.<sup>36,37</sup> Consistent with our previous findings with PGE<sub>2</sub>,<sup>27</sup> PGE<sub>1</sub> administration suppressed circulating IL-12 levels, but increased the IL-10 production after *E. coli*

TABLE 1. Effect of PGE<sub>1</sub> on the Absolute Number of Liver Lymphocytes After *E. coli* Infection

Cell Fraction	Naive	24 Hours		36 Hours	
		PGE <sub>1</sub>	α-CD	PGE <sub>1</sub>	α-CD
CD3 <sup>int</sup> IL-2Rβ <sup>+</sup> (NKT)	29.1 $\pm$ 4.1	22.8 $\pm$ 2.8*	35.7 $\pm$ 3.3	21.2 $\pm$ 3.5*	40.6 $\pm$ 1.8
CD3 <sup>+</sup> IL-2Rβ <sup>-</sup> (T)	12.2 $\pm$ 4.9	25.7 $\pm$ 3.6	21.0 $\pm$ 2.5	24.6 $\pm$ 4.1	22.8 $\pm$ 2.6
CD3 <sup>-</sup> IL-2Rβ <sup>+</sup> (NK)	35.2 $\pm$ 6.5	35.2 $\pm$ 5.9	38.9 $\pm$ 4.8	42.5 $\pm$ 7.4	48.1 $\pm$ 3.2
αβ T cell	30.8 $\pm$ 7.2	38.2 $\pm$ 4.7	44.1 $\pm$ 3.8	37.2 $\pm$ 7.4	51.5 $\pm$ 5.9
γδ T cell	7.6 $\pm$ 2.3	8.3 $\pm$ 2.5	10.5 $\pm$ 3.2	9.8 $\pm$ 3.2	13.0 $\pm$ 2.9

NOTE. Data are expressed as means  $\pm$  SD ( $\times 10^4$ ). C3H/HeN mice were inoculated with  $1 \times 10^8$  CFU/mouse of *E. coli* 3 hours after PGE<sub>1</sub> or α-CD treatment. Liver lymphocytes were obtained at indicated times after infection and the absolute numbers of each population were calculated by multiplying the whole numbers by the percentages as assessed by FACS analysis.

\* $P < .05$  versus the control α-CD-treated group.

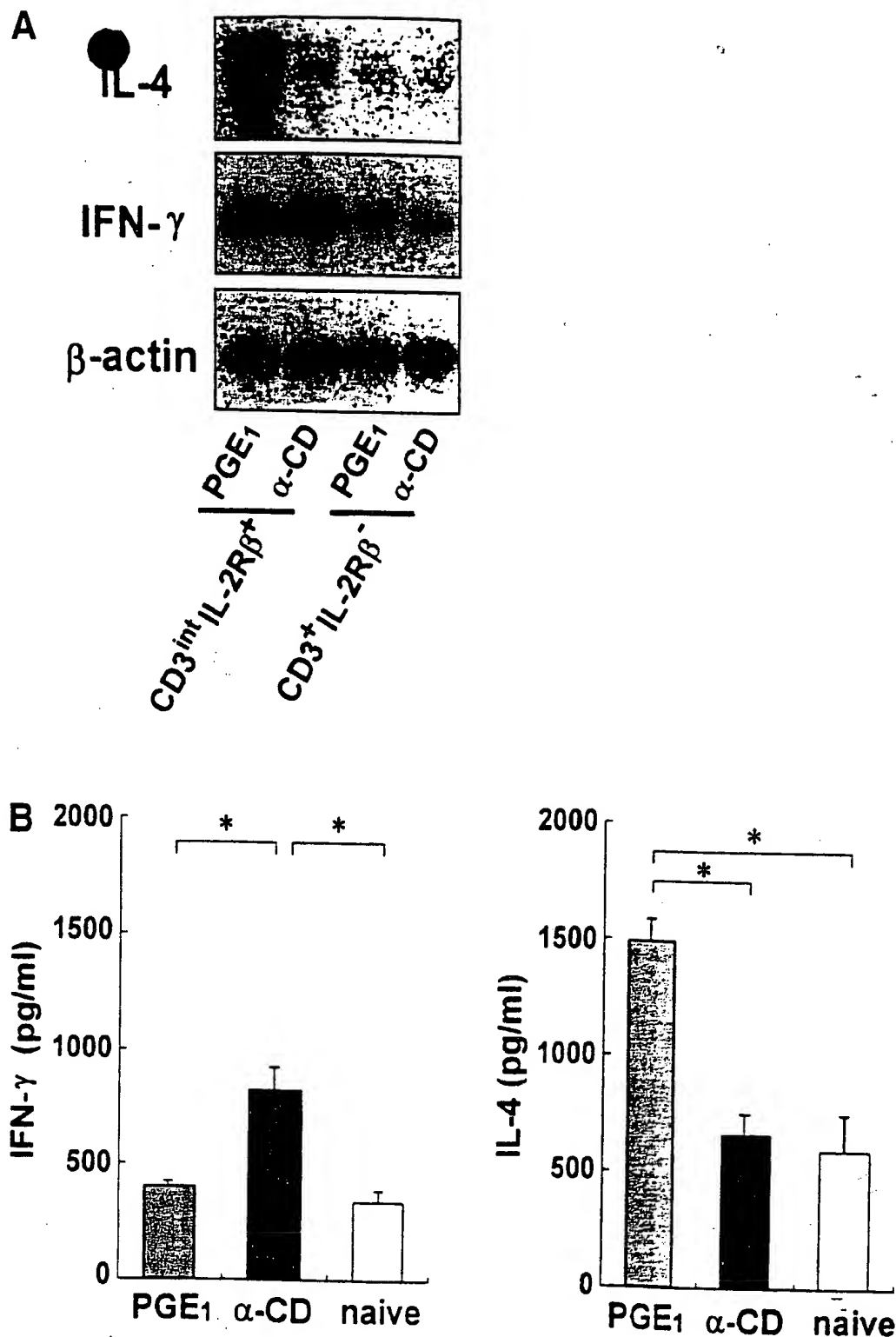


FIG. 5. IL-4 and interferon gamma production by liver lymphocytes. (A) Expression of cytokine mRNA in liver CD3<sup>int</sup> IL-2R $\beta$ <sup>+</sup> cells and CD3<sup>+</sup> IL-2R $\beta$ <sup>-</sup> cells sorted from mice infected with *E. coli*. Mice were infected IP with  $1.0 \times 10^8$  CFU of *E. coli* (1/5 LD<sub>50</sub>) 3 hours after the administration of 87.5 mg/kg of PGE<sub>1</sub> or  $\alpha$ -CD. Twenty-four hours after infection, CD3<sup>int</sup> IL-2R $\beta$ <sup>+</sup> cells and CD3<sup>+</sup> IL-2R $\beta$ <sup>-</sup> cells were sorted from liver lymphocytes pooled from 5 mice of each group, and total RNA was isolated for reverse transcription-PCR. (B) IL-4 and interferon gamma production by liver lymphocytes on TCR stimulation. Liver lymphocytes ( $5 \times 10^5$ /well) from mice infected with *E. coli* 3 hours after the administration of 87.5 mg/kg of PGE<sub>1</sub> or  $\alpha$ -CD 24 hours previously were incubated in anti-CD3 $\epsilon$  mAb-coated 96-well plates for 48 hours at 37°C and the culture supernatants were collected. The cytokine activity in the culture supernatant was determined for the presence of interferon gamma or IL-4 by ELISA. Data are representative of 3 separate experiments and are expressed as the means of triplicates  $\pm$  SD in an experiment. \* $P < .05$ .

challenge. The modulation of inflammatory and anti-inflammatory cytokine production may be responsible for the suppressive effect of PGE<sub>1</sub> on liver injury. However, unlike PGE<sub>2</sub>, TNF- $\alpha$  production in *E. coli*-infected mice was not affected by PGE<sub>1</sub> pretreatment. We do not know at present why PGE<sub>1</sub> does not inhibit TNF- $\alpha$  production induced by *E. coli* infection. PGE<sub>1</sub> and PGE<sub>2</sub> equally bind to EP<sub>2</sub> and EP<sub>3</sub>, which mediate the inhibitory effect on TNF- $\alpha$  production, whereas PGE<sub>1</sub> binds less efficiently to EP<sub>1</sub>, which mediates Ca<sup>2+</sup> increase.<sup>9,38-40</sup> Such differences may be related to the different effects of PGE<sub>1</sub> and PGE<sub>2</sub> on TNF- $\alpha$  production following *E. coli* infection. However, this is only speculation,

and further investigation is needed to confirm this speculation.

The unique liver T cells CD3<sup>int</sup> IL-2R $\beta$ <sup>+</sup> T cells are now known to correspond to NK T cells.<sup>23-26</sup> NK T cells are found in the thymus,<sup>41</sup> liver,<sup>42</sup> and bone marrow,<sup>43</sup> where they account for populations of CD4<sup>+</sup> CD8<sup>-</sup> and CD4<sup>+</sup> CD8<sup>-</sup> TCR  $\alpha\beta$  cells. A large number of NK T cells express an invariant TCR encoded by the V $\alpha$ 14 and J $\alpha$ 281 gene segments<sup>23,26</sup> and are selected by the non-polymorphic MHC class I-like surface protein CD1d.<sup>23</sup> NK T cells in the liver have recently been found to be an essential target of IL-12 for interferon production and cytotoxicity by an NK-like effector mecha-

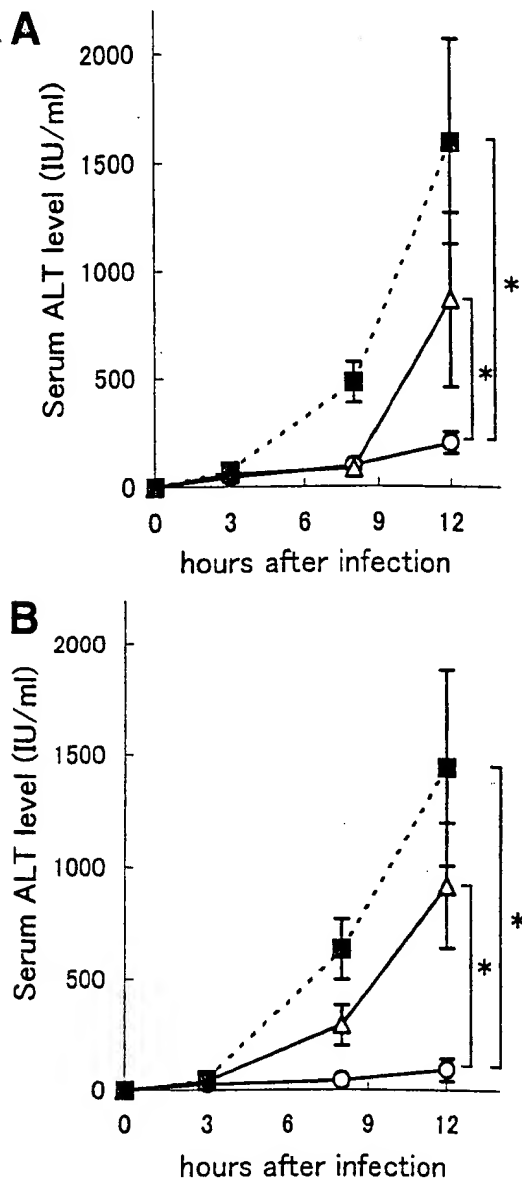


FIG. 6. Effect of anti-IL-4 mAb or anti-IL-10 mAb on inhibitory effect of PGE<sub>1</sub> on *E. coli*-induced liver injury. (A) *E. coli* ( $1.0 \times 10^9$  CFU/mouse;  $2 \times \text{LD}_{50}$ ) was injected IP 3 hours after administration of 87.5 mg/kg of PGE<sub>1</sub> and 10 mg/kg of control rat IgG1 Ab, PGE<sub>1</sub> and 10 mg/kg of anti-IL-4 Ab or  $\alpha$ -CD in PBS. (O), PGE<sub>1</sub> plus control mAb; ( $\Delta$ ), PGE<sub>1</sub> plus anti-IL-4 mAb; ( $\blacksquare$ ),  $\alpha$ -CD. (B) *E. coli* ( $1.0 \times 10^9$  CFU/mouse;  $2 \times \text{LD}_{50}$ ) was injected IP 3 hours after the administration of 87.5 mg/kg of PGE<sub>1</sub> and 10 mg/kg of control rat IgM Ab, PGE<sub>1</sub> and 10 mg/kg of anti-IL-10 mAb or  $\alpha$ -CD in PBS. Sera were collected from mice after *E. coli* challenge and measured for ALT activities. Data are representative of 3 separate experiments and are expressed as the mean  $\pm$  SD for 5 mice in an experiment. \* $P < .05$ . (O), PGE<sub>1</sub> plus control mAb; ( $\Delta$ ), PGE<sub>1</sub> plus anti-IL-10 mAb; ( $\blacksquare$ ),  $\alpha$ -CD.

nism.<sup>44</sup> Therefore, it is possible that NK T cells in the liver may contribute to LPS-induced liver injury via cytokine production and/or direct cytotoxicity against hepatocytes. We have recently found that  $\beta 2$ -microglobulin<sup>-/-</sup> or  $\alpha 281$ <sup>-/-</sup> mice were resistant to liver injury induced by *Salmonella* infection, suggesting that NK T cells expressing a canonical TCR V $\alpha 14/\alpha 281$  may play a critical role in the occurrence of liver injury after *Salmonella* infection.<sup>45</sup> Infection with *E. coli* induces a marked increase in IL-12 production by macrophages<sup>27,46</sup> but administration of PGE<sub>1</sub> decreased IL-12 production and concurrently inhibited the Th1-like response of liver T cells and liver injury after *E. coli* challenge. Taken

together, these results strongly suggest that IL-12-activated NK T cells in the liver may play a pivotal role in the occurrence of LPS-induced liver injury after *E. coli* infection, and inhibition of the emergence of NK T cells in liver, and more importantly, inhibition of the Th1-like response and cytotoxic activity of such liver T cells may partly account for the inhibitory effect of PGE<sub>1</sub> on liver injury.

In contrast to liver injury after *E. coli* infection, bacterial clearance after infection was impaired by PGE<sub>1</sub> administration. Inflammatory response, including a Th1-like response, is important for control of infection with various pathogens.<sup>47,48</sup> Inhibition of the Th1-like response of liver T cells is partly responsible for impairment of the host defense against *E. coli* infection in PGE<sub>1</sub>-treated mice.

A notable finding in the present study is that early IL-4 production is potentially involved in protection of PGE<sub>1</sub> against liver injury after *E. coli* infection. Rapid IL-4 secretion by NK T cells has been shown in mice treated with anti-CD3 mAb.<sup>49,50</sup> NK T cells rapidly produce large amounts of IL-4 on binding of the  $\beta 2$ -microglobulin-associated CD1d.<sup>23</sup> In the present study, IL-4 production was detected in the liver T cells stimulated *in vitro* with immobilized anti-CD3 mAb, and it was significantly augmented in PGE<sub>1</sub>-treated mice. Although IL-4 production was not detected in the serum of either control mice or PGE<sub>1</sub>-treated mice after *E. coli* infection, *in vivo* administration of anti-IL-4 mAb significantly decreased the inhibitory effect of PGE<sub>1</sub> on *E. coli*-induced liver injury. IL-4 may be secreted in a very small amount, thus acting in a narrow intracellular range in the liver. NK T cells have been reported to specialize in recognizing glycosylceramides containing  $\alpha$ -anomeric sugar with a longer fatty acyl chain and sphingosine base, which are detected in certain bacteria but rarely detected in normal mammalian tissues.<sup>51</sup> Although it is not known whether *E. coli* bacteria have the relevant antigens, NK T cells may respond to the related antigens derived from *E. coli* and produce IL-4 rapidly, which in turn controls inflammatory responses after *E. coli* infection in PGE<sub>1</sub>-treated mice. How can IL-4 protect against liver injury? The production of proinflammatory cytokines such as IL-12 in monocytes/macrophages is regulated by deactivating cytokines such as IL-4, IL-13, IL-10, and transforming growth factor  $\beta$ .<sup>36,37,52,53</sup> In fact, our results showed that administration of anti-IL-10 mAb diminished the inhibitory effect of PGE<sub>1</sub> on the liver injury. Therefore, it is possible that IL-4 in synergy with IL-10 may suppress the macrophage activity for production of inflammatory cytokines. Interferon gamma is known to be a potent stimulator for Fas-Fas ligand-mediated apoptosis and perforin induction in NK and T cells,<sup>54</sup> offering an alternative possibility that early IL-4 in synergy with IL-10 may regulate the interferon gamma production and cytotoxic activity of NK and T cells, resulting in suppression of liver injury induced by *E. coli* infection.

Naive Th cells initially stimulated in the presence of IL-12 and interferon gamma tend to develop into Th1 cells secreting IL-2, interferon gamma, and TNF- $\beta$  for induction of cell-mediated immunity characterized by macrophage activation and cytotoxic T lymphocyte induction, whereas IL-4 promotes differentiation of Th2 cells secreting IL-4, IL-5, IL-6, and IL-10 for assisting in antibody production.<sup>47,48</sup> Our results show that administration of PGE<sub>1</sub> decreased IL-12 production, but enhanced IL-10 release in the serum after *E. coli* challenge. The modulation of cytokine production by PGE<sub>1</sub> in the liver may be responsible for the change of liver T



cells to a Th-2-like response after *E. coli* infection. Furthermore, it has been reported that PGE<sub>2</sub>, as well as other agents that increase cAMP, inhibits IL-12R $\beta$  expression and consequently IL-12 responsiveness of T cells.<sup>55</sup> PGE<sub>2</sub> profoundly decreases production of IL-2 and interferon gamma by Th1 cells but enhances IL-4 production by Th2 cells.<sup>1,16</sup> Therefore, it is also possible that PGE<sub>1</sub> directly binds to receptors for PGEs, such as EP<sub>2</sub> and EP<sub>4</sub>, on liver T cells and shifts the Th1/Th2 balance to a Th2-like response through enhancement of intracellular cAMP.

In conclusion, PGE<sub>1</sub> affects liver injury after *E. coli* infection through modulating the Th1/Th2 balance in liver T cells. Our data provide an insight into the prophylactic approach to control liver injury associated with infection with Gram-negative bacteria.

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# Failure of ketoprofen and interferon combination therapy to improve interferon-resistant chronic hepatitis C

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FH Anderson, L Zeng, EM Yoshida, NR Rock. Failure of ketoprofen and interferon combination therapy to improve interferon-resistant chronic hepatitis C. *Can J Gastroenterol* 1997;11(4):294-297. Preliminary reports suggest that patients with interferon (IFN)-resistant chronic hepatitis C respond better to a combination of IFN- $\alpha$  and nonsteroidal anti-inflammatory drugs than to IFN alone. The efficacy of IFN combined with ketoprofen in the treatment of patients with IFN-resistant chronic hepatitis C was evaluated. Seventeen patients, nonresponsive after at least six months of treatment with IFN- $\alpha$ 2b and subsequently treated with the combination of IFN- $\alpha$ 2b plus ketoprofen for four months, were studied. Serum aminotransferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and serum hepatitis C virus (HCV) RNA were analyzed before and throughout treatment. No patient normalized serum aminotransferases after combination therapy. There were no significant differences in mean serum ALT and AST levels before and after ketoprofen intervention. Serum HCV RNA became undetectable after treatment in only one patient, but was detectable again three months after treatment cessation. These results provide no convincing evidence that the combination of IFN- $\alpha$ 2b with ketoprofen improves the response to IFN in patients nonresponsive to IFN alone.

**Key Words:** Combination therapy, Hepatitis C, Interferon, Ketoprofen

## Échec d'un traitement associatif au kétoprofène et à l'interféron dans les cas d'hépatite C chronique résistante à l'interféron

**RÉSUMÉ :** Selon des résultats préliminaires, les patients souffrant d'hépatite C chronique résistante à l'interféron (IFN) répondent mieux à une association d'IFN- $\alpha$  et d'anti-inflammatoires non stéroïdiens par rapport à l'IFN seul. L'efficacité de l'IFN associé au kétoprofène dans le traitement des patients souffrant d'hépatite C chronique résistante à l'IFN a été évaluée. Sept patients qui ne répondaient pas après au moins six mois de traitement à l'IFN- $\alpha$ 2b et par la suite traités au moyen d'une combinaison d'IFN- $\alpha$ 2b plus kétoprofène pendant quatre mois ont été examinés. Les aminotransférases sériques (alanine aminotransférase [ALT] et aspartate aminotransférase [AST]) et l'ARN sérique du virus de l'hépatite C (HCV) ont été analysés avant, puis tout au long du traitement. Les aminotransférases sériques ne se sont normalisées chez aucun des patients après le traitement associatif. Aucune différence significative n'a été notée quant aux taux sériques moyens d'ALT et d'AST avant et après l'administration de kétoprofène. L'ARN du HCV sérique est devenu indétectable après le traitement chez un seul patient, mais était à nouveau décelable trois mois après la fin du traitement. Ces résultats n'offrent aucune preuve concluante que le traitement associatif IFN- $\alpha$ 2b et kétoprofène améliore la réponse à l'IFN chez les patients qui ne répondent pas à l'IFN seul.

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Over the past few years interferon (IFN)-alpha has been widely used to treat chronic infection with hepatitis C virus (HCV). A short term response, however, is seen in only approximately 40% to 50% of treated patients (1,2), and the percentage of patients achieving a long term response is significantly less. In a recent multicentre study only 22% of patients treated with IFN for 18 months were able to maintain a long term response, as reflected by normal serum aminotransferase, more than 18 months after discontinuing therapy (2). To improve the efficacy of IFN therapy, numerous strategies employing adjuvant therapy have been proposed. Medications such as ursodeoxycholic acid and ribavirin have recently been reported to result in an improved long term response rate when administered with IFN (3-5).

Certain nonsteroidal anti-inflammatory drugs (NSAIDs), which act as cyclooxygenase inhibitors, reportedly increase the bioavailability of IFN. An in vitro study demonstrated that indomethacin amplifies transduction of the IFN postreceptor signal, leading to an increased biosynthesis of serum 2'5'-oligoadenylate synthetase, an IFN-induced enzyme with antiviral activity (6). As well, an increase in serum 2'5'-oligoadenylate synthetase has been observed in patients with chronic viral hepatitis following indomethacin administration (7).

Recently, small studies have reported that NSAIDs including ketoprofen can improve the response to IFN in IFN-resistant chronic hepatitis C patients (8-10). In our practice, however, we did not observe such a favourable response. We thus reviewed our experience using ketoprofen plus IFN in chronic hepatitis C patients unresponsive to standard IFN therapy.

#### PATIENTS AND METHODS

Office charts of all patients seen in the authors' hepatitis clinic were reviewed. Patients with chronic HCV infection who failed to have biochemical and virological responses to the following treatment were identified: IFN- $\alpha$ 2b (INTRON A, Schering Canada Inc)  $3 \times 10^6$  U subcutaneously three times weekly for at least six consecutive months; then ketoprofen (ORUDIS, Rhône-Poulenc Rorer) 100 mg orally tid for four months while continuing IFN at the same dose. A nonresponse to IFN was defined as failure to normalize serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST), as well as the presence of HCV viremia after at least six months of IFN therapy. Any patient who had interrupted IFN therapy for any period was excluded from the analysis. Any patient in whom ketoprofen was discontinued secondary to adverse side effects before the four-month study period was completed was also excluded from analysis.

Diagnosis of chronic hepatitis C was based on persistent elevations of serum aminotransferases for at least six months; the presence of antibody to HCV (second-generation enzyme-linked immunoassay); and exclusion of other causes of chronic liver diseases.

During therapy with IFN alone and with ketoprofen plus IFN, serum ALT and AST were monitored monthly accord-

ing to routine clinic protocol. The monthly mean ALT and AST levels of all patients were calculated during the initial treatment with IFN alone and during the four months of ketoprofen plus IFN. Serum ALT and AST levels at the end of IFN therapy and the combination therapy were individual compared for each patient. The presence of serum HCV RNA was determined before initiating IFN therapy and was repeated at three-month intervals throughout treatment. The primers for polymerase chain reaction (PCR) were selected from the 5'-noncoding region of the HCV genome based on a method previously described (11).

Statistical analysis was by the paired *t* test to determine significance between serum ALT and AST levels after IFN alone and after combination therapy. The 95% CI levels were computed. Data are expressed as mean  $\pm$  SD when appropriate.

#### RESULTS

From 1994 to 1995, patients with chronic hepatitis C were treated at the authors' hepatitis clinic with ketoprofen plus IFN if they failed to improve after at least six months of IFN alone. Criteria for enrolment in this combination therapy were a well-defined chronic hepatitis secondary to HCV infection, absence of evidence for human immunodeficiency virus and hepatitis B infections, and exclusion of other causes of chronic liver diseases. Twenty-one patients who were unresponsive to IFN alone and who were then treated with ketoprofen plus IFN were identified. These patients were given the combination therapy without selection in any way.

One patient was lost to follow-up after one month of combination therapy, and three others discontinued ketoprofen secondary to gastrointestinal intolerance after one ( $n=2$ ) or two ( $n=1$ ) months of use, respectively. None of these four patients had normal ALT or AST levels when the ketoprofen plus IFN therapy was ended. The remaining 17 patients completed four months of combination therapy and were included in the analysis. Fifteen patients were treated with the combination therapy immediately after failure to respond to the six-month IFN therapy. One patient was treated with the combination therapy after nine consecutive months of treatment with IFN alone and the other after 11 consecutive months. For these two patients, the six months of treatment with IFN alone was matched to that of the other 15 by using the ALT and AST levels tested in the last six months of treatment with IFN alone.

Thirteen of the 17 patients analyzed were male. Patient age ranged from 40 to 59 years (mean 47). Eleven patients had a known parenteral source of infection (ie, blood transfusions or intravenous drug use); the other six had no identifiable source. A liver biopsy performed in all patients before IFN treatment revealed chronic active hepatitis with piecemeal necrosis in five patients, chronic active hepatitis with cirrhosis in nine and chronic active hepatitis with bridging fibrosis in three. All patients had compensated liver disease. Serum HCV RNA was detected in all but one patient before IFN treatment. This patient had histology compatible with

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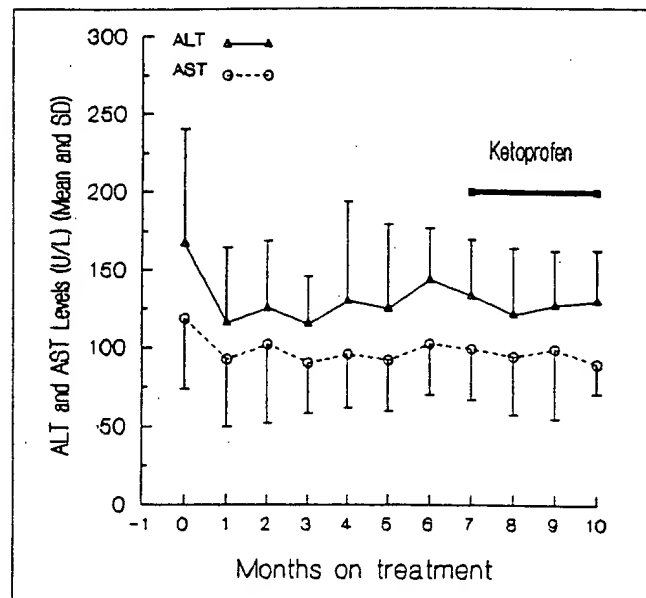
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**TABLE 1**  
**Clinical summary of patients before interferon treatment**

Number of patients	17
Male:female ratio	13:4
Age range (years)	40 to 59 (mean=47)
Alanine aminotransferase (U/L) (mean $\pm$ SD)*	152 $\pm$ 75
Aspartate aminotransferase (U/L) (mean $\pm$ SD) <sup>†</sup>	115 $\pm$ 45
Antihepatitis C virus (+)	17
Hepatitis C virus RNA (+)	16
Hepatitis B surface antigen (-)	17
Antihuman immunodeficiency virus (-)	17
Other causes of liver diseases	0
Risk factors of infection	
Blood transfusion	6
Intravenous drug use	5
Unknown source	6
Liver histology	
Chronic active hepatitis only	5
Chronic active hepatitis with cirrhosis	9
Chronic active hepatitis with bridging fibrosis	3

\*Normal range: 10 to 55 U/L; <sup>†</sup>Normal range: 19 to 38 U/L



**Figure 1** Monthly serum levels (mean  $\pm$  SD) of the aminotransferases (alanine aminotransferase [ALT] and serum aspartate aminotransferase [AST]) of all 17 patients during the treatment period. Normal range of serum ALT is 10 to 55 U/L, and of AST is 19 to 38 U/L. The bar identifies the months of ketoprofen intervention.

chronic hepatitis C on liver biopsy (12), although a further recombinant immunoblot assay was not done and no other causes of chronic liver diseases were found. Mean serum ALT and AST of all patients before treatment with IFN alone was 167 $\pm$ 73 U/L and 115 $\pm$ 45 U/L, respectively. Patient characteristics are summarized in Table 1.

Mean serum ALT after six months of IFN alone was 144 $\pm$ 33 U/L (range 96 to 198), and after four months of ketoprofen plus IFN was 130 $\pm$ 33 U/L (range 87 to 202). Mean serum AST after six months of IFN alone was 102 $\pm$ 33 U/L (range 70 to 178), and after four months of ketoprofen plus IFN was 90 $\pm$ 19 U/L (range 61 to 122). Serum ALT (normal range 10 to 55 U/L) and serum AST (normal range 19 to 38 U/L) did not normalize in any patient after ketoprofen plus IFN therapy. The monthly mean ALT and AST levels for all patients in the entire study period are shown in Figure 1. In the present self-paired study, there was no significant difference in either mean serum ALT or AST level ( $P>0.1$  for both) before and after the addition of ketoprofen. The 95% CI for the mean differences in serum ALT and AST levels were -3.7 to 37.5 U/L and 13 to 26.8 U/L, respectively.

Serum HCV RNA remained positive in 15 of the 17 patients throughout the 10-month treatment. For the remaining two patients, serum HCV RNA was not detected before and throughout the study in one. In the other, PCR became negative after combined therapy, but his serum ALT and AST failed to normalize. Three months after discontinuation of ketoprofen plus IFN, his PCR result was positive again.

A liver biopsy was available in only one patient after ketoprofen plus IFN treatment. Pretreatment histology revealed cirrhosis with moderately severe chronic hepatitis in this patient; however, a repeated liver biopsy one month after completing ketoprofen plus IFN demonstrated an unchanged histological feature.

## DISCUSSION

Our results have shown that there is no benefit in using the combination of ketoprofen with IFN in patients with chronic hepatitis C in whom IFN alone has failed. None of our patients, including four who had only one to two months of treatment, was biochemically responsive to the combination therapy. There was no significant difference in either mean serum ALT or AST before and after ketoprofen intervention. After ketoprofen intervention there was only one patient in whom ALT and AST dropped - from 177 to 87 U/L and from 66 to 50 U/L, respectively. This patient, however, had previously demonstrated a drop in enzymes on two occasions during treatment with IFN alone (ALT dropped from 203 to 78 and 79 U/L, and AST from 113 to 55 and 67 U/L). Thus, the enzyme drop observed in this patient may not have resulted from ketoprofen. Rather, it may be a feature of fluctuated aminotransferase in chronic hepatitis C.

Serum HCV RNA became undetectable in one patient after combination therapy; however, his PCR result was positive again three months after completing therapy. There was, therefore, no long term suppression of HCV viremia in any patient.

Hannigan and Williams (6) originally demonstrated that IFN postreceptor signal transduction in vitro may be mediated via the products of arachidonic acid metabolism, and that the cyclooxygenase and lipoxygenase inhibitors enhanced

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sion. Inhibition of cyclooxygenase and lipoxygenase, the  
two most well known arms of the arachidonic acid metabolic  
pathway, may allow more arachidonic acid to be oxidized by  
a third pathway, possibly epoxygenase-mediated, which pro-  
duces an IFN second messenger. Indomethacin, a cyclo-  
oxygenase inhibitor, has been demonstrated by Andreone et  
al (7) to enhance the production of IFN-induced antiviral  
enzymes.

Ketoprofen, an NSAID with the ability to inhibit both  
cyclooxygenase and lipoxygenase, has been reported in ab-  
stracts, again by Andreone et al (8,9), to have a beneficial ef-  
fect in combination with IFN to treat refractory chronic  
hepatitis C patients who were originally nonresponders to  
IFN alone. Our experience with the paired design reported  
here, however, contrasts markedly with these favourable re-  
sults; we were unable to document a beneficial response,  
either biochemically or virologically. As an NSAID, keto-  
profen has a chemical structure different from indomethacin

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for his assistance in preparing the graphics and Debra Sussman for  
her assistance in chart review.

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(13). Although our study failed to demonstrate a beneficial response to ketoprofen, we cannot exclude the possibility that other NSAIDs in combination with IFN may be of value.

The presence of cirrhosis, which may render a lower re-  
sponse rate to IFN, in nine of the 17 patients may have ob-  
scured a possible beneficial effect of ketoprofen (14). These  
nine patients, however, were all clinically compensated and  
probably reflective of many patients with chronic hepatitis C  
referred for IFN therapy. Our experience is most likely a fair  
representation of the IFN-unresponsive population for  
whom adjuvant therapy is desirable.

## CONCLUSIONS

Our study provides no convincing evidence that the combi-  
nation of IFN with ketoprofen improves the response to IFN  
in patients nonresponsive to IFN alone. More studies are re-  
quired to confirm the efficacy of the combination of IFN  
with NSAIDs to treat refractory chronic hepatitis C.

# Tenoxicam, a Non-Steroid Anti-Inflammatory Drug, Is Unable To Increase the Response Rate in Patients With Chronic Hepatitis C Treated by Alpha Interferon

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The purpose of this study is to compare a combination of interferon (IFN)- $\alpha_2a$  (Roferon) + Tenoxicam with IFN- $\alpha_2a$  alone in the treatment of chronic hepatitis C. This prospective, randomized double-blind study included 149 patients, all of whom were diagnosed with active chronic hepatitis C but non-cirrhotic (ALT  $\geq 1.5$  upper limit of normal, anti-hepatitis C virus (HCV) positive by enzyme-linked immunosorbent assay<sub>2</sub> and RIBA<sub>3</sub>). The patients were randomized in two groups, as follows: G1 (n = 76): IFN- $\alpha_2a$  3 million units times per week during 6 months + placebo; and G2 (n = 73): IFN- $\alpha_2a$  3 million units three times per week + Tenoxicam (20 mg/day) during 6 months. Alanine aminotransferase (ALT) and HCV RNA were determined before and at months 6 and 12 of treatment. 2'5' oligoadenylate synthetase activity (2'5' AS) was dosed in mononuclear cells before and at 3-month treatment intervals in 28 patients. Liver biopsy was performed before and 6 months after the end of therapy. Parameters were similar before therapy for both groups. Biochemical and virological responses were similar for both groups at month 6 (49.3% vs. 42.9% and 43.3% vs. 38.3%, respectively) and month 12 (28.3% vs. 23.8% and 17.2% vs. 17.5%, respectively). HCV RNA level significantly decreased in both groups at month 6, with no difference whatever the therapy; however, the HCV RNA level returned to initial values at month 12 and was the only significant prognostic factor of a sustained response. No peak of 2'5' AS activity was observed during treatment in patients with dual therapy. A histological improvement was also noted in both groups without difference, regardless of therapy. The percentage of adverse events was identical for both groups. Paracetamol intake, assessed in 80 patients, was 49.1 g per 6 months in the G1

group and 22.5 g per 6 months in the G2 group (not significant). In conclusion, the non-steroid anti-inflammatory drug, Tenoxicam, does not increase IFN $\alpha$  efficacy in the treatment of chronic hepatitis C. This combination is well tolerated and partially lowers Paracetamol intake, but not preexisting  $\alpha$ -IFN adverse events. (HEPATOLOGY 1998;27:862-867.)

Chronic hepatitis C is a common and worldwide cause of liver cirrhosis and hepatocellular carcinoma.<sup>1</sup> Interferon alpha (IFN $\alpha$ ) is currently used for the treatment of chronic hepatitis C.<sup>2</sup> A common regimen of 3 million units of interferon alpha (IFN $\alpha$ ) three times weekly for 24 weeks can normalize serum alanine aminotransferase (ALT) level in 40% to 50% of the patients during treatment.<sup>3,4</sup> Nevertheless, the sustained ALT and HCV RNA response rates are usually inferior to 25%.<sup>4</sup> Finding the optimal treatment to induce long-term biochemical and virological remission in patients with chronic hepatitis C remains a great challenge. To improve the response rate, several investigators attempted to administer IFN in combination with other drugs. An interesting approach involves inhibition of prostaglandin synthesis and activation of arachidonic acid metabolism.<sup>5</sup> *In vitro*, the treatment of cells with IFN $\alpha$  induces hydrolysis of phosphatidylcholine by phospholipase A<sub>2</sub>, which releases arachidonic acid. Thus, phospholipase A<sub>2</sub> activation occurs as an early event in the IFN $\alpha$  response and is selectively involved in the activation of a transcription factor complex, as follows: IFN-stimulated gene factor three.<sup>6</sup> Arachidonic acid is metabolized by cyclo-oxygenase into prostaglandins or by lipoxygenase into leukotrienes. Both prostaglandins and leukotrienes serve as specific second messengers. The inhibition of the cyclo-oxygenase and/or lipoxygenase pathways by a non-steroid anti-inflammatory drug blocks prostaglandin production,<sup>7</sup> has been shown to amplify the IFN signal, and has been shown to increase the concentration of 2'5' oligoadenylate synthetase (2'5' AS), which is an enzyme associated with the antiviral activity of IFN.<sup>8</sup> In preliminary reports on small pilot studies, Andréone et al.<sup>9,10</sup> showed that the addition of a non-steroid anti-inflammatory drug (Indomethacin [Indocid; Merck Sharp et Dhome-Chibret, NJ] or Ketoprofen [Profenid; Rhone-Poulenc, Rorer, Paris, France]) enhanced the efficacy of IFN in some initially nonresponder patients. Tenoxicam is a non-steroid anti-inflammatory drug and is one of the oxicam derivatives, which is used in the

Abbreviations: IFN $\alpha$ , interferon alpha; HCV, hepatitis C virus; 2'5' AS, 2'5' oligoadenylate synthetase; ALT, alanine aminotransferase.

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management of inflammatory diseases. Its potency is very close to that of indomethacin and other oxicams as an inhibitor of prostaglandin biosynthesis *in vitro*.<sup>11</sup> This controlled, randomized double-blind pilot study was initiated to assess and compare the effect and tolerance of a Tenoxicam and IFN $\alpha$  combination, with those of IFN $\alpha$  alone in the treatment of chronic hepatitis C.

## PATIENTS AND METHODS

### Patients

One hundred and seventy-one patients were enrolled in a multicentric, double-blind trial between 1993 and 1995, according to the following criteria: age range between 18 and 60 years; baseline ALT above or equal to 1.5 times the upper normal limit level, which was reached twice during the last 6 months; hepatitis B surface antigen and antibody to human immunodeficiency virus negative; antibody to HCV (anti-HCV) positive by enzyme-linked immunosorbent assay<sub>2</sub> and RIBA<sub>3</sub>; histological chronic hepatitis data through liver biopsy taken within the preceding year; and neither prior treatment by IFN nor contra-indication to non-steroid anti-inflammatory drug therapy. Patients with the following were excluded: with histological findings of apparent cirrhosis, neurological dysfunction, chronic renal failure, malignancy, active alcoholism, and causes of liver disease other than that by HCV; a hemoglobin count < 10 g/dL; a platelet count < 75,000/mm<sup>3</sup>; a granulocyte count < 1,500/mm<sup>3</sup>; and failure to provide written informed consent.

### Methods

**Study Protocol.** Within each center, patients were randomly assigned to be treated with 3 million units of subcutaneous IFN $\alpha_2a$  (Roferon Roche, Basel, Switzerland) three times weekly during 6 months and Tenoxicam (20 mg/d *per os* to be taken with a meal for 6 months) or with a placebo (identical tablets). Group 1 received IFN $\alpha_2a$  + placebo and group 2 received IFN $\alpha_2a$  + Tenoxicam. The patients were monitored for 1 year. Patients were seen monthly during the treatment period and for an additional 6-month period of follow-up after the treatment was stopped. Compliance was monitored by evaluating the regularity of attendance at clinical visits, by reviewing patient diaries, and by pill count. Paracetamol intake was evaluated and expressed as g per 6 months. Adverse events were evaluated at each visit. Tenoxicam only was stopped when patients developed presumed Tenoxicam-adverse events; on the other hand, both IFN $\alpha_2a$  and Tenoxicam were stopped when patients developed presumed IFN $\alpha$ -adverse events. The study protocol was approved by the Grenoble Hospital Ethics Committee, Grenoble, France. Written informed consent was obtained from each patient before enrollment.

**Laboratory Assays.** Clinical and laboratory assessments were performed at each visit and included routine biochemical, hematological, and liver function tests. Commonly available enzyme immunoassays were used to determine serum hepatitis B surface antigen, human immunodeficiency virus 1 antibodies anti-human immunodeficiency virus 1 (Abbott Laboratory, North Chicago, IL), and anti-HCV antibodies (EIA-2 Ortho Diagnostic Systems, Racitan, NJ, and Abbott Laboratories). HCV RNA was detected in serum with a diagnostic polymerase chain reaction assay (Amplicor HCV™, Roche Molecular Systems). HCV RNA was also quantified in the pretreatment serum specimens with a quantitative polymerase chain reaction method (Amplicor™ HCV and Monitor™, Roche Diagnostic Systems, Basel, Switzerland). The Monitor™ lower threshold was calculated to be 800 copies/mL.<sup>12-14</sup> HCV serotype was detected with an enzyme immunoassay for antibodies directed to peptides encoded by the NS4 region of the HCV genome. These

peptides are specific of serotypes 1, 2, 3, 4, 5, and 6 (Murex Diagnostics, Chatillon, France). The assay is performed in micro-wells coated with serotype specific HCV antigen.<sup>15</sup>

**Dosage of 2'5' AS Activity.** The 2'5' AS activity level was measured in 28 nonselected consecutive patients treated in one center (13 from the Tenoxicam + IFN $\alpha_2a$  group, 15 from the IFN $\alpha_2a$  group) on peripheral blood mononuclear cells at months 1, 3, 6, 9, and 12. Peripheral blood mononuclear cells were isolated from venous blood by Ficoll-Hypaque gradient centrifugation. Cells were lysed in a buffer containing 0.5% NP40 (10<sup>7</sup> cells/mL). The enzyme assay was performed as previously described.<sup>15</sup> Standard incubation conditions were as described. Briefly, cell supernatants were first incubated with poly (rI). (rC) agarose beads. The washed beads were then incubated (2 h at 37°C) in the presence of 50 g/mL poly (rI). (rC) in a buffer (pH 8) containing 3 mmol/L adenosine triphosphate and [<sup>32</sup>P]-adenosine triphosphate. Aliquots of the bead supernatants were spotted on a PEI-cellulose thin-layer plate. After overnight chromatography, the spots containing ATP and the 2'5' AS were cut out and counted.

The specific activity of 2'5' AS is defined as 1 U per 10<sup>7</sup> cells (one enzyme unit = 1 nmol adenosine monophosphate residues incorporated into 2'5' AS per minutes at 37°C).<sup>16</sup>

**Liver Histology.** Liver biopsy was performed in all patients 1 to 12 months before treatment. A second liver biopsy was offered to all patients at 12 months but was accepted by only 117 patients. However, a single independent observer blindly scored all liver specimens on 79 patients only. The histological changes of the liver were graded by histology activity index of the Knodell scoring system<sup>17</sup> and by the Metavir scoring system.<sup>18</sup> Histological improvement was defined as a decrease of at least one point in the score, for histological activity between first and second liver biopsies.

**Assessment of Efficacy.** A complete response was defined as those patients who developed complete normalization of serum ALT levels and whose tests were negative for HCV RNA at the completion of the 6-month treatment protocol. Sustained response was defined as the persistence of normal serum ALT levels and as negative testing for HCV RNA, 6 months after the end of therapy.

**Statistical Analysis.** Treatment results were based on an intent-to-treat analysis, as follows: no patient was excluded after randomization, even in the case of non-compliance or missing data. At least 93 patients were required to detect a 20% difference in the biochemical response between treatments in each group, assuming that with the standard treatment (IFN $\alpha_2a$ ), 50% or more of the patients would show normal ALT values during the 6 months of treatment (type-I error = 0.05; and type-II error = 0.20). The results were expressed as arithmetic means ( $\pm$  one SD) and percentages. The non-parametric Mann-Whitney test was used to compare qualitative variables. Quantitative data was compared with the Student *t* test. Univariate analysis was carried out first to identify the factors associated with the complete and sustained biochemical response. A multivariate analysis was then used to identify independent factors associated with responses. In the stepwise, logistic regression model, explanatory variables were those that were significantly related to responses in the univariate model (*P* < .05). Univariate and multivariate analyses completed with statistical software (SPSS statistical software, Statistical Product and Science Solution, SPSS Inc., Chicago, IL).

## RESULTS

Of the 171 patients enrolled in the study, 22 were excluded from statistical analysis because of protocol deviation; thus, 149 patients were eligible for the study. Eleven patients were lost to follow-up. At the onset of the study, relevant epidemiological, biochemical, virological, and histological features were similar between the 2 groups (Table 1).

TABLE 1. Baseline Characteristics of the Two Groups of Patients

Characteristics	IFN $\alpha_2$ a (n = 76)	NSAID-IFN $\alpha_2$ a (n = 73)
<b>General</b>		
Age (yr)	37.5 $\pm$ 11.0	37.3 $\pm$ 9.8
Body mass index (W [kg]/S [cm] <sup>2</sup> )	0.024 $\pm$ 0	0.024 $\pm$ 0
Gender (M/F)	50/26	56/17
Duration since exposure (y)	9.9 $\pm$ 5.9	10.3 $\pm$ 6.1
Probable exposure (%)		
Transfusion	28.9	24.7
IVDA	44.7	43.8
Other	25	34.2
Unknown	14.5	24.7
<b>Biochemical and virological</b>		
ASAT (ULN)	1.9 $\pm$ 1.3	2.3 $\pm$ 2.8
ALAT (ULN)	3.6 $\pm$ 2.5	4.5 $\pm$ 5.4
Alcaline phosphatases (ULN)	0.6 $\pm$ 0.3	0.7 $\pm$ 0.3
GGT (ULN)	1.4 $\pm$ 1.3	1.7 $\pm$ 1.8
Bilirubinemia ( $\mu$ mol/L)	11.3 $\pm$ 10.4	13.4 $\pm$ 15.8
TP (%)	96 $\pm$ 6	97 $\pm$ 11
Ferritinemia ( $\mu$ g/L)	213.1 $\pm$ 200.3	252.2 $\pm$ 227.3
IgG (g/l)	15 $\pm$ 6	13 $\pm$ 6
IgA (g/L)	2 $\pm$ 1	2 $\pm$ 1
IgM (g/L)	2 $\pm$ 1	1 $\pm$ 1
Beta-2-microglobulin	2.2 $\pm$ 1.1†	2.3 $\pm$ 1.3*
HCV RNA level (10 <sup>3</sup> genomes/mL)	3.3 $\pm$ 4.6‡	2.3 $\pm$ 2.8§
Serotype (%)		
1/2/3	52.9/8.6/17.1	50.8/13.8/20.0
4/5/6	1.4/1.4/—	6.2/1.5/—
Mixed	8.6	—
Undetermined	7.7	10.0

Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; Ig, immunoglobulin; W, weight (kg); S, size (cm); ULN, upper limit of normal.

\*n = 51.

†n = 54.

‡n = 60.

§n = 58.

### Biochemical and Virological Response

The percentage of patients who achieved complete and sustained biochemical responses was statistically identical in both groups. ALT levels were significantly lower at months 6 and 12 for both groups. The percentage of patients with undetectable HCV RNA by PCR in serum was also similar at

months 6 and 12 (Table 2). The HCV RNA level significantly decreased in both groups at month 6 with no statistic difference. However, HCV RNA level returned to initial value at month 12 for both groups. No significant difference was found between the two groups when the percentage of normal ALT patients and HCV RNA clearance was compared, both during and after treatment.

### Histological Response

The evolution of histological lesions is presented in Table 3. A decrease of the Knodell score and of the Metavir index "activity" parameter ( $P < .05$ ) was observed in both groups. Within the Knodell score, only the periportal necrosis score significantly decreased ( $P < .05$ ). Other parameters did not change.

### 2'5' AS

The characteristics of patients assessed for 2'5' AS were similar in the 2 specific groups (Table 4). A significant peak of 2'5' AS activity level was observed only in the IFN $\alpha_2$ a group at month 3 ( $P < .05$ ). In the IFN $\alpha_2$ a + Tenoxicam group, no difference was observed between day 1 and month 3. A relationship was noted between serotype and peak of 2'5' AS at month 3. Indeed, 2'5' AS activity level reached  $6.23 \pm 5.58$  at month 3 in the serotype 1 group (vs.  $0.55 \pm 2.27$  at day 1) ( $P < .05$ ); it reached  $2.62 \pm 3.16$  in the serotype non 1 group (vs.  $1.47 \pm 2.38$ ) (non significant). The 2'5' AS activity level returned to its initial value at month 6 and 12, for these 2 groups.

### Statistical Analysis

For the univariate analysis, the pretreatment variables significantly associated with a complete biochemical response were as follows: normal GGT activity ( $P = .013$ ); low HCV RNA level ( $P = .04$ ); low body-mass index ( $P = .009$ ); and serotype non 1 ( $P = .036$ ). Only low HCV RNA ( $P = .002$ ) and serotype non 1 ( $P = .017$ ) were still significantly associated with normal ALT at month 12. The results of the multivariate analysis show that the sustained biochemical response was associated with only one pretreatment parameter: low HCV RNA ( $P = .045$ ).

TABLE 2. Biochemical and Virological Results in the Two Groups of Patients

	IFN $\alpha_2$ a (n = 76)			IFN $\alpha_2$ a + Tenoxicam (n = 73)		
	Day 1	Month 6	Month 12	Day 1	Month 6	Month 12
Normal serum ALT	0	35/71 (49.3%)	17/60 (28.3%)	0	30/70 (42.9%)	15/63 (23.8%)
ALT level (ULN) (mean $\pm$ 1 SD)	3.6 $\pm$ 2.5	2.0 $\pm$ 2.6*	2.6 $\pm$ 2.4†‡	4.5 $\pm$ 5.4	1.8 $\pm$ 1.8*	2.7 $\pm$ 2.4§#
HCV RNA negative	0	26/60 (43.3%)	10/58 (17.2%)	0	23/60 (38.3%)	10/57 (17.5%)
HCV RNA level (10 <sup>3</sup> Eq Genomes/mL) (mean $\pm$ 1 SD)	3.3 $\pm$ 4.6	1.2 $\pm$ 2.7*	3.1 $\pm$ 4.5†	2.3 $\pm$ 2.8	1.9 $\pm$ 3.7##	3.7 $\pm$ 5.4**

Abbreviation: ULN, upper limit of normal.

\* $P < .001$  (between Day 1 and month 6).

† $P < .005$  (between month 6 and month 12).

‡ $P < .001$  (between month 1 and month 12).

§ $P < .001$  (between month 6 and month 12).

# $P < .002$  (between month 1 and month 12).

## $P < .02$  (between month 1 and month 6).

\*\* $P < .008$  (between month 6 and month 12).



TABLE 3. Evolution of Histological Lesions in the Two Groups of Patients

	IFN $\alpha_2a$ (n = 40)			IFN $\alpha_2a$ + Tenoxicam (n = 39)		
	Before	After	Improvement*	Before	After	Improvement*
Knodell score	6.8 $\pm$ 2.6	5.7 $\pm$ 2.6†	26 (65%)	7.6 $\pm$ 3.0	6.6 $\pm$ 3.0†	20 (51%)
Periportal necrosis	1.9 $\pm$ 1.4	1.4 $\pm$ 1.1†	20 (50%)	2.1 $\pm$ 1.2	1.5 $\pm$ 1.2†	16 (41%)
Focal necrosis	1.0 $\pm$ 0.7	0.8 $\pm$ 0.8	13 (32.5%)	1.3 $\pm$ 0.9	1.2 $\pm$ 0.9	8 (20.5%)
Periportal inflammation	3.0 $\pm$ 1.0	2.7 $\pm$ 1.1	14 (35%)	3.0 $\pm$ 1.0	2.8 $\pm$ 1.0	10 (25.6%)
Fibrosis	0.9 $\pm$ 0.6	0.9 $\pm$ 0.7	4 (10%)	1.2 $\pm$ 0.9	1.2 $\pm$ 0.9	3 (7.7%)
Metavir index						
Activity	1.5 $\pm$ 0.9	1.1 $\pm$ 0.7†	20 (50%)	1.5 $\pm$ 0.8	1.2 $\pm$ 0.7†	16 (41%)
Fibrosis	1.4 $\pm$ 0.9	1.4 $\pm$ 0.9	7 (17.5%)	1.6 $\pm$ 0.9	1.7 $\pm$ 0.9	3 (8%)

\*Percentage of patients with histological improvement of at least one grade.

†P &lt; .05.

### Adverse Events

The most common adverse events are presented in Table 5. No difference was observed between the two groups for usual adverse events of Interferon and Tenoxicam therapy. Twelve patients stopped Tenoxicam or placebo + IFN $\alpha_2a$  treatment caused by adverse events (7 in the IFN $\alpha_2a$  group and 5 in IFN $\alpha_2a$  + Tenoxicam group). Thirteen patients only stopped Tenoxicam or placebo (5 in IFN $\alpha_2a$  group and 8 in IFN $\alpha_2a$  + Tenoxicam group) because of adverse events. However, two patients presented a gastric peptic ulcer requiring omeprazole treatment. The number of patients who did not complete therapy was similar in both groups. The number of paracetamol pills taken during the study and evaluated for 80 patients was 49 g/6 months in the IFN $\alpha_2a$  group and 22 g/6 months in the IFN $\alpha_2a$  + Tenoxicam group (non significant).

### DISCUSSION

This study shows low rates of sustained response despite administration of Tenoxicam to improve treatment outcome. The 17% sustained complete response rate in patients with

chronic hepatitis C treated by IFN  $\alpha$  correlates with previous observations on similar patients following IFN $\alpha$  therapy.<sup>3,4</sup>

It has been recently suggested<sup>5,19</sup> that the administration of a non-steroid anti-inflammatory drug could increase the *in vitro* response rate in nonresponder chronic hepatitis C patients. Until now, only small pilot studies have been carried out in nonresponder patients<sup>20-22</sup> using various non-steroid anti-inflammatory drugs (Sulindac, Indomethacin, and Piroxicam). No or partial biochemical response to treatment was detectable. 2'5' AS serum activity increased, especially during the first 2 weeks of treatment. Only one small randomized controlled trial was performed in HCV chronic hepatitis patients non-responder to IFN $\alpha$  alone. A combination of IFN $\alpha$  plus Ketoprofen in comparison with IFN $\alpha$  alone or IFN $\alpha$  plus ribavirin<sup>10</sup> was used in this trial. An ALT normalization was observed in 37% of the cases at months 3 and 4 in the IFN $\alpha$  + Ketoprofen group as compared to 6% in the IFN $\alpha$  group.

The present study is the first to be carried out on a large series of patients and to take into account both virological and histological parameters in addition to biochemical variables. The response rate for dual therapy was lower than that observed by Andréone et al.<sup>10</sup> and not statistically different from that of the IFN $\alpha$  group, however the selected non-steroid anti-inflammatory drug, the inclusion criteria, and the characteristics of patients at enrollment were different. Indeed the non-steroid anti-inflammatory drug used was Tenoxicam, selected for its pharmacological properties. The drug is completely bioavailable after oral intake. Its long half-life

TABLE 4. Main Characteristics of Patients for Whom 2'5' AS Was Measured

	IFN $\alpha_2a$ (n = 15)	IFN $\alpha_2a$ + Tenoxicam (n = 13)
Age†	36 $\pm$ 12	32 $\pm$ 9
Gender (M/F)	10/5	10/3
ALT (ULN)†	5.7 $\pm$ 3.5	6.6 $\pm$ 10.1
Serotype (Number)		
1/2/3	8/1/3	6/2/2
4/5/6	2/1/1	3
Undetermined	1	
HCV RNA† (10 <sup>5</sup> Eq/Génomes/mL)	2.3 $\pm$ 2.9	2.7 $\pm$ 3.4
Knodell score†	6.0 $\pm$ 2.0	7.2 $\pm$ 1.6
Biochemical response (%)		
Month 6	53%	38%
Month 12	13%	7%
2'5' AS†		
Day 1	0.67 $\pm$ 0.30	1.13 $\pm$ 2.26
Month 3	6.08 $\pm$ 5.44*	2.85 $\pm$ 3.48
Month 6	2.33 $\pm$ 1.85	2.19 $\pm$ 2.85
Month 9	1.17 $\pm$ 1.32	0.49 $\pm$ 0.31
Month 12	1.09 $\pm$ 0.77	0.82 $\pm$ 0.01

\*P &lt; .05 (between month 3 and day 1).

†Mean  $\pm$  1 SD.‡1 UL/10<sup>7</sup> cells.

TABLE 5. Adverse Events in the Two Groups of Patients

	IFN $\alpha_2a$ (n = 76)	IFN $\alpha_2a$ + Tenoxicam (n = 73)
IFN		
Asthenia	67 (88%)	62 (85%)
Flu-like syndrome	51 (67%)	48 (66%)
Depression	13 (18%)	15 (20%)
Leucopenia	4 (5%)	1 (1%)
Thrombocytopenia	1 (1%)	1 (1%)
Hypothyroidism	1 (1%)	1 (1%)
Other adverse events	5 (7%)	8 (11%)
Tenoxicam/Placebo		
Epigastric pain	22 (30%)	24 (32%)
Peptic ulcer	—	2 (3%)
Diarrhea	19 (25%)	23 (32%)
Skin rash	18 (24%)	18 (25%)

allows the administration of a single and well-tolerated daily dose. Tenoxicam, even though it has favorable pharmacological properties, has not been tested for its ability to enhance IFN $\alpha$  responsiveness *in vitro*. Tenoxicam has been previously tested in chronic hepatitis B.<sup>23</sup> In the quoted report, the combination was well tolerated and increased the effectiveness of IFN $\alpha$  at the end of therapy, leading to the disappearance of HBV DNA in 8 of 11 (73%) patients. However, it is possible that oxycams, such as Tenoxicam, exert a lower inhibition of cyclo-oxygenase/lipo-oxygenase activity. However, as far as we know, the comparison of various non-steroid anti-inflammatory drugs efficacy had never been made for chronic hepatitis C.

Bias, caused by the greater number of unknown contamination in the Tenoxicam + IFN $\alpha$  group, was unlikely because clinical and biochemical findings did not statistically differ according to whether the patients did or did not have anti-inflammatory drug. Co-infections were found in 8.6% of the cases in the IFN $\alpha$  group, but global serotype distribution was not statistically different in both groups.

In this study, no 2'5'AS peak was observed in the IFN $\alpha$  + Tenoxicam group contrary to the results of Andreone et al.<sup>8</sup> Previous studies have shown enhanced levels of 2'5' AS activity in serum and peripheral blood mononuclear cells of chronic hepatitis C patients, treated by dual therapy, especially in responder patients.<sup>24,25</sup> The peak seemed to vary from one patient to another and to be related to the dose of IFN $\alpha$  and to the virus genotype. The peak seemed to be unrelated to the duration of treatment.<sup>26</sup> In most studies, 2'5' AS was tested in serum<sup>7</sup> or in supernatant after culture of blood mononuclear cells.<sup>8</sup> Thus, it appears that the conditions of 2'5' AS dosage are probably very important. It also seems preferable to dose 2'5' AS in mononuclear cells, at a given time, after drug intake.<sup>9</sup>

The only predictive factor of sustained response was low HCV RNA level. This result is similar to those observed in recent therapeutic studies.<sup>27</sup> Serotype was not associated with a better response, contrary to other studies. Possible explanations for such a discrepancy include the following: 1) the absence of dissociation between subtype 1a and 1b; and 2) the number of undetermined sera, in both cases caused by the method used.

No serious adverse events were observed in the dual therapy group, except for two peptic ulcers. The prevalence of gastric symptoms was similar between the two groups and not different from that usually observed in non-steroid anti-inflammatory drug tolerance studies.<sup>28</sup> Dual therapy patients had a lower rate of paracetamol intake, which might indicate a probable improvement of IFN $\alpha$  tolerance, typically in the case of the flu-like syndrome. The peptic ulcers were not complicated but required omeprazole treatment. Andreone et al.<sup>10</sup> suggested that in case of dual therapy, all patients should be medicated with sucralfate or anti-H<sub>2</sub>-receptor antagonists to ensure gastroprotection against non-steroid anti-inflammatory drug mucosal damage.

In conclusion, in this controlled randomized trial, Tenoxicam was not found to increase response rate to IFN $\alpha$  in the treatment of chronic hepatitis C in naive patients. It could be interesting to compare various non-steroid anti-inflammatory drugs because some drugs may have a stronger cyclo-oxygenase/lipo-oxygenase inhibitory effect. The present study suggests that the inhibitory effect of these drugs if any is probably low.

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Ratio of Rates vs. Placebo	95% CI on Ratio of Rates
0.41	(0.16, 1.05)
0.74	(0.33, 1.64)
2.79	(1.47, 5.30)

Ratio of Rates vs. Placebo	95% CI on Ratio of Rates
1.04	(0.36, 3.01)
1.73	(0.65, 4.61)
5.72	(2.36, 13.89)

**(GI) Effects—Risk of GI Ulceration, Bleeding**

estestinal toxicity such as bleeding, ulceration of the stomach, small intestine, or colon may occur at any time, with or without warning, in patients treated with nonsteroidal anti-inflammatory drugs (NSAIDs). Minor upper gastrointestinal symptoms such as dyspepsia, are common and may occur during NSAID therapy. Therefore, patients should remain alert for ulceration, bleeding, or other signs of GI toxicity and the steps to take if they occur. Periodic laboratory monitoring has not been adequately assessed, nor has it been adequately assessed whether patients who develop a serious upper GI ulcer while on NSAID therapy is symptomatic. It is noted that upper GI ulcers, gross bleeding, or perforation appear to occur in approximately 1% to 3% of patients treated for 3–6 months, and in about 1% to 3% of patients treated for 6 months to 1 year. These trends continue, and the likelihood of developing a serious upper GI ulcer during the course of therapy. However, therapy is not without risk.

**CLINICAL STUDIES, Special Studies, In Patients with Osteoarthritis**

Among 337 patients who received VIOXX in controlled clinical trials of 1-year duration (most were enrolled in 12-month studies) at a daily dose of 12.5 mg to 50 mg, 33% experienced a serious upper GI event. Two patients experienced a serious upper GI event within three months (at day 62 and 87, respectively). One additional patient experienced a serious upper GI event within six months (Day 130) and the remaining 30% of upper GI bleed within 12 months. Of these 33%, 23% of these 33% patients required them to be free of ulceration. It is unclear if this study population is representative of the general population. Prospective, long-term studies are needed to compare the incidence of serious upper GI adverse events in patients taking VIOXX with those in patients taking other NSAID products have not been performed.

**Precautions**

Patients should be prescribed with extreme caution in patients with a prior history of ulcer disease or gastrointestinal bleeding. Most spontaneous reports of fatal GI bleeding or debilitated patients and therefore, extreme caution is taken in treating this population. The potential risk for an adverse GI event, the lower the risk, the more likely the patient should be used for the shortest possible duration. In high-risk patients, alternate therapies should be considered. Patients should be considered for NSAIDs should be considered for patients with a prior history of ulcer disease and/or gastrointestinal bleeding. Patients with a greater than 10-fold higher risk of bleeding than patients with neither of these conditions. In addition to a past history of ulcer disease, epidemiological studies have identified other risk factors or co-morbid conditions that may increase the risk for GI bleeding such as: treatment with anticoagulants, treatment with antiplatelet agents, longer duration of therapy, smoking, alcoholism, older age, and poor health status.

**Reactions**

In general, anaphylactoid reactions in patients without known prior exposure to NSAIDs, rare cases of angioedema and angioedema have been reported in patients taking VIOXX. VIOXX should not be given to patients with a history of severe allergic reactions to aspirin triad. This symptom complex includes asthma, rhinitis, and nasal polyps, or who exhibit severe

fatal bronchospasm or taking aspirin or other NSAIDs (see CONTRAINDICATIONS and PRECAUTIONS, Preexisting Asthma). Emergency help should be sought in cases where an anaphylactoid reaction occurs. **Renal Disease**—Safety information is available regarding the use of VIOXX in patients with advanced kidney disease. There is no information on the use of VIOXX in patients with advanced kidney disease. Therefore, treatment with VIOXX is not recommended in these patients. If VIOXX therapy must be initiated, close monitoring of the patient's kidney function is advisable (see PRECAUTIONS, Renal Effects).

**Precautions**

VIOXX cannot be expected to substitute for corticosteroids to treat corticosteroid insufficiency. Abrupt discontinuation of corticosteroids may lead to exacerbation of corticosteroid-responsive illness. Patients on prolonged corticosteroid therapy should have their therapy tapered slowly if a decision is made to discontinue corticosteroids. The pharmacological activity of VIOXX in reducing inflammation, and possibly fever, may diminish the utility of these diagnostic signs in detecting infectious complications of presumed noninfectious, painful conditions.

**Effects**

Line elevations of one or more liver tests may occur in up to 15% of patients taking NSAIDs, and notable elevations of ALT or AST (approximately three or more times the upper limit of normal) have been reported in approximately 1% of patients in clinical trials with NSAIDs. These laboratory abnormalities may progress, may remain unchanged, or may be transient with continuing therapy. Rare cases of severe hepatic reactions, including jaundice and fatal fulminant hepatitis, liver necrosis and hepatic failure (some with a fatal outcome) have been reported with NSAIDs. In controlled clinical trials of VIOXX, the incidence of borderline elevations of liver tests at doses of 12.5 and 25 mg daily was comparable to the incidence observed with ibuprofen and less than that observed with diclofenac. In placebo-controlled trials, approximately 0.5% of patients taking rofecoxib (12.5 or 25 mg QD) and 0.1% of patients taking placebo had notable elevations of ALT or AST.

Patients with symptoms and/or signs suggesting liver dysfunction, or in whom an abnormal liver test has occurred, should be monitored carefully for evidence of the development of a more severe hepatic reaction while on therapy with VIOXX. Use of VIOXX is not recommended in patients with moderate or severe hepatic insufficiency (see Pharmacokinetics, Special Populations). If clinical signs and symptoms consistent with liver disease develop, or if systemic manifestations occur (e.g., eosinophilia, rash, etc.), VIOXX should be discontinued.

**Effects**

Long-term administration of NSAIDs has resulted in renal papillary necrosis and other renal injury. Renal toxicity has also been seen in patients in whom renal prostaglandins have a compensatory role in the maintenance of renal perfusion. In these patients, administration of a nonsteroidal anti-inflammatory drug may cause a dose-dependent reduction in prostaglandin formation and, secondarily, in renal blood flow, which may precipitate overt renal decompensation. Patients at greatest risk of this reaction are those with impaired renal function, heart failure, liver dysfunction, taking diuretics and ACE inhibitors, and the elderly. Continuation of NSAID therapy is usually followed by recovery to the pretreatment state. Clinical trials with VIOXX at daily doses of 12.5 and 25 mg have shown renal effects (hypertension, edema) similar to those observed with comparator NSAIDs; these occur with an increased frequency with chronic use of VIOXX at doses above the 12.5 to 25 mg range. (See ADVERSE REACTIONS.)

**Precautions**

VIOXX should be used when initiating treatment with VIOXX in patients with considerable dehydration. It is advisable to rehydrate patients first and then start therapy with VIOXX. Caution is also recommended in patients with preexisting kidney disease (see WARNINGS, Advanced Renal Disease).

**Effects**

Anemia is sometimes seen in patients receiving VIOXX. In placebo-controlled trials, there were no significant differences observed between VIOXX and placebo in clinical results of anemia. Patients on long-term treatment with VIOXX should have their hemoglobin or hematocrit checked if they exhibit any signs or symptoms of anemia or blood loss. VIOXX does not generally affect platelet counts, prothrombin time (PT), or partial thromboplastin time (PTT). VIOXX does not inhibit platelet aggregation at indicated doses (see CLINICAL STUDIES, Special Studies, Platelets).

**Retention and Edema**

Retention and edema have been observed in some patients taking VIOXX (see ADVERSE REACTIONS). VIOXX should be used with caution, and should be introduced at the lowest recommended dose in patients with fluid retention, hypertension, or heart failure.

**Asthma**

Patients with asthma may have aspirin-sensitive asthma. The use of aspirin in patients with aspirin-sensitive asthma has been associated with severe bronchospasm which can be fatal. Since cross reactivity, including bronchospasm, between aspirin and other nonsteroidal anti-inflammatory drugs has been reported in such aspirin-sensitive patients,

VIOXX should not be administered to patients with this form of aspirin sensitivity and should be used with caution in patients with preexisting asthma.

**Information for Patients**

VIOXX can cause discomfort and, rarely, more serious side effects, such as gastrointestinal bleeding, which may result in hospitalization and even fatal outcomes. Although serious GI tract ulcerations and bleeding can occur without warning symptoms, patients should be alert for the signs and symptoms of ulcerations and bleeding, and should ask for medical advice when observing any indicative signs or symptoms. Patients should be apprised of the importance of this follow-up (see WARNINGS, Gastrointestinal (GI) Effects—Risk of GI Ulceration, Bleeding and Perforation).

Patients should promptly report signs or symptoms of gastrointestinal ulceration or bleeding, skin rash, unexplained weight gain, or edema to their physicians.

Patients should be informed of the warning signs and symptoms of hepatotoxicity (e.g., nausea, fatigue, lethargy, pruritus, jaundice, right upper quadrant tenderness, and "flu-like" symptoms). If these occur, patients should be instructed to stop therapy and seek immediate medical attention.

Patients should also be instructed to seek immediate emergency help in the case of an anaphylactoid reaction (see WARNINGS).

In late pregnancy VIOXX should be avoided because it may cause premature closure of the ductus arteriosus.

**Laboratory Tests**

Because serious GI tract ulcerations and bleeding can occur without warning symptoms, physicians should monitor for signs or symptoms of GI bleeding.

**Drug Interactions**

**ACE Inhibitors:** Reports suggest that NSAIDs may diminish the antihypertensive effect of Angiotensin-Converting Enzyme (ACE) inhibitors. In patients with mild to moderate hypertension, administration of 25 mg daily of VIOXX with the ACE inhibitor benazepril, 10 to 40 mg for 4 weeks, was associated with an average increase in mean arterial pressure of about 3 mm Hg compared to ACE inhibitor alone. This interaction should be given consideration in patients taking VIOXX concomitantly with ACE inhibitors.

**Aspirin:** Concomitant administration of low-dose aspirin with VIOXX may result in an increased rate of GI ulceration or other complications, compared to use of VIOXX alone. At steady state, VIOXX 50 mg once daily had no effect on the anti-platelet activity of low-dose (81 mg once daily) aspirin, as assessed by *ex vivo* platelet aggregation and serum TXB<sub>2</sub> generation in clotting blood. VIOXX is not a substitute for aspirin for cardiovascular prophylaxis.

**Cimetidine:** Co-administration with high doses of cimetidine (800 mg twice daily) increased the C<sub>max</sub> of rofecoxib by 21%, the AUC<sub>0-12h</sub> by 23% and the t<sub>1/2</sub> by 15%. These small changes are not clinically significant and no dose adjustment is necessary.

**Digoxin:** Rofecoxib 75 mg once daily for 11 days does not alter the plasma concentration profile or renal elimination of digoxin after a single 0.5 mg oral dose.

**Furosemide:** Clinical studies, as well as post-marketing observations, have shown that NSAIDs can reduce the natriuretic effect of furosemide and thiazides in some patients. This response has been attributed to inhibition of renal prostaglandin synthesis.

**Ketoconazole:** Ketoconazole 400 mg daily did not have any clinically important effect on the pharmacokinetics of rofecoxib.

**Lithium:** NSAIDs have produced an elevation of plasma lithium levels and a reduction in renal lithium clearance. Thus, when VIOXX and lithium are administered concurrently, subjects should be observed carefully for signs of lithium toxicity.

**Methotrexate:** VIOXX 75 mg administered once daily for 10 days increased plasma concentrations by 23% as measured by AUC<sub>0-24h</sub> in patients receiving methotrexate 7.5 to 15 mg/week for rheumatoid arthritis. An equivalent magnitude of reduction in methotrexate renal clearance was observed. At 24 hours postdose, a similar proportion of patients treated with methotrexate alone (94%) and subsequently treated with methotrexate co-administered with 75 mg of rofecoxib (88%) had methotrexate plasma concentrations below the measurable limit (5 ng/mL). The effects of the recommended doses for osteoarthritis (12.5 and 25 mg) of VIOXX on plasma methotrexate levels are unknown. Standard monitoring of methotrexate-related toxicity should be continued if VIOXX and methotrexate are administered concomitantly.

**Oral Contraceptives:** Rofecoxib did not have any clinically important effect on the pharmacokinetics of ethinyl estradiol and norethindrone.

**Prednisone/prednisolone:** Rofecoxib did not have any clinically important effect on the pharmacokinetics of prednisone or prednisolone.

**Rifampin:** Co-administration of VIOXX with rifampin 600 mg daily, a potent inducer of hepatic metabolism, produced an approximate 50% decrease in rofecoxib plasma concentrations. Therefore, a starting daily dose of 25 mg of VIOXX should be considered for the treatment of osteoarthritis when VIOXX is co-administered with potent inducers of hepatic metabolism.

**Warfarin:** Anticoagulant activity should be monitored, particularly in the first few days after initiating or changing VIOXX therapy in patients receiving warfarin or similar agents, since these patients are at an increased risk of bleeding complications. In single and multiple dose studies

in healthy subjects receiving prothrombin time (measured approximately 8% to 11%). I bleeding events have been reported in elderly, in association with patients receiving VIOXX or Carcinogenesis, Mutagenesis: Rofecoxib was not carcinogenic to 30 mg/kg (male) and 60 mg/kg (female) in the human exposure AUC<sub>0-24h</sub> and in male and female mice (approximately 10 mg/kg daily at 25 and 50 mg daily in Rofecoxib was not mutagenic in mammalian cell mutagenesis (CHO) cells, in an *in vitro* assay, or in an *in vivo* mouse bone marrow. Rofecoxib did not impair mouse up to 100 mg/kg (approximately 10 mg/kg daily in Rofecoxib had no effect on fertility 30 mg/kg (approximately 10 mg/kg daily in Rofecoxib was not teratogenic in rats and rabbits (approximately 25- and 50 mg daily based on non-statistically significant of vertebral malformations mg/kg/day (approximately 25 and 50 mg daily based on in pregnant women. VIOXX nancy only if the potential to the fetus.

**Nonteratogenic effects:** Rofecoxib was not teratogenic in rats and rabbits (approximately 25- and 50 mg daily based on non-statistically significant of vertebral malformations mg/kg/day (approximately 25 and 50 mg daily based on in pregnant women. VIOXX nancy only if the potential to the fetus.

**Labor and delivery:** Rofecoxib produced no evidence of labor or parturition in rats and rabbits (approximately 10- and 30 mg/kg daily based on the AUC<sub>0-24h</sub> at 25 and 50 mg/kg daily in pregnant Merck & Co., Inc. maintains nancy outcomes of women pregnant. Healthcare providers prenatal exposure to VIOXX during pregnancy should be avoided.

**Nursing mothers**

Rofecoxib is excreted in milk in rats and rabbits (approximately 10- and 30 mg/kg daily based on the AUC<sub>0-24h</sub> at 25 and 50 mg/kg daily in pregnant Merck & Co., Inc. maintains nancy outcomes of women pregnant. Healthcare providers prenatal exposure to VIOXX during pregnancy should be avoided.

**Pediatric Use**

Safety and effectiveness of 18 years have not been established.

**Geriatric Use**

Of the patients who received clinical trials, 1455 were 65 years of age or older. In safety and effectiveness studies, subjects and younger subjects and older individuals cannot be distinguished. In one of these studies (clinical trial), VIOXX

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## Cel brex—Cont.

month studies) at a daily dose of 200 mg or more, 2 (0.04%) experienced significant upper GI bleeding, at 14 and 22 days after initiation of dosing. Approximately 40% of these 5,285 patients were in studies that required them to be free of ulcers by endoscopy at study entry. Thus it is unclear if this study population is representative of the general population. Prospective, long-term studies required to compare the incidence of serious, clinically significant upper GI adverse events in patients taking CELEBREX vs. comparator NSAID products have not been performed.

NSAIDs should be prescribed with extreme caution in patients with a prior history of ulcer disease or gastrointestinal bleeding. Most spontaneous reports of fatal GI events are in elderly or debilitated patients and therefore special care should be taken in treating this population. To minimize the potential risk for an adverse GI event, the lowest effective dose should be used for the shortest possible duration. For high risk patients, alternate therapies that do not involve NSAIDs should be considered.

Studies have shown that patients with a prior history of peptic ulcer disease and/or gastrointestinal bleeding and who use NSAIDs, have a greater than 10-fold higher risk for developing a GI bleed than patients with neither of these risk factors. In addition to a past history of ulcer disease, pharmacoepidemiological studies have identified several other co-therapies or co-morbid conditions that may increase the risk for GI bleeding such as: treatment with oral corticosteroids, treatment with anticoagulants, longer duration of NSAID therapy, smoking, alcoholism, older age, and poor general health status.

**Anaphylactoid Reactions**

As with NSAIDs in general, anaphylactoid reactions have occurred in patients without known prior exposure to CELEBREX. In post-marketing experience, rare cases of anaphylactoid reactions and angioedema have been reported in patients receiving CELEBREX. CELEBREX should not be given to patients with the aspirin triad. This symptom complex typically occurs in asthmatic patients who experience rhinitis with or without nasal polyps, or who exhibit severe, potentially fatal bronchospasm after taking aspirin or other NSAIDs (see CONTRAINDICATIONS and PRECAUTIONS—Preexisting Asthma). Emergency help should be sought in cases where an anaphylactoid reaction occurs.

**Advanced Renal Disease**

No information is available regarding the use of CELEBREX in patients with advanced kidney disease. Therefore, treatment with CELEBREX is not recommended in these patients. If CELEBREX therapy must be initiated, close monitoring of the patient's kidney function is advisable (see PRECAUTIONS—Renal Effects).

**Pregnancy**

In late pregnancy CELEBREX should be avoided because it may cause premature closure of the ductus arteriosus.

**Familial Adenomatous Polyposis (FAP):** Treatment with CELEBREX in FAP has not been shown to reduce the risk of gastrointestinal cancer or the need for prophylactic colectomy or other FAP-related surgeries. Therefore, the usual care of FAP patients should not be altered because of the concurrent administration of CELEBREX. In particular, the frequency of routine endoscopic surveillance should not be decreased and prophylactic colectomy or other FAP-related surgeries should not be delayed.

**PRECAUTIONS**

**General:** CELEBREX cannot be expected to substitute for corticosteroids or to treat corticosteroid insufficiency. Abrupt discontinuation of corticosteroids may lead to exacerbation of corticosteroid-responsive illness. Patients on prolonged corticosteroid therapy should have their therapy tapered slowly if a decision is made to discontinue corticosteroids. The pharmacological activity of CELEBREX in reducing inflammation, and possibly fever, may diminish the utility of these diagnostic signs in detecting infectious complications of presumed noninfectious, painful conditions.

**Hepatic Effects:** Borderline elevations of one or more liver tests may occur in up to 15% of patients taking NSAIDs, and notable elevations of ALT or AST (approximately three or more times the upper limit of normal) have been reported in approximately 1% of patients in clinical trials with NSAIDs. These laboratory abnormalities may progress, may remain unchanged, or may be transient with continuing therapy. Rare cases of severe hepatic reactions, including jaundice and fatal fulminant hepatitis, liver necrosis and hepatic failure (some with fatal outcome) have been reported with NSAIDs, including CELEBREX. (See ADVERSE REACTIONS—post-marketing experience.) In controlled clinical trials of CELEBREX, the incidence of borderline elevations of liver tests was 6% for CELEBREX and 5% for placebo, and approximately 0.2% of patients taking CELEBREX and 0.3% of patients taking placebo had notable elevations of ALT and AST.

A patient with symptoms and/or signs suggesting liver dysfunction, or in whom an abnormal liver test has occurred, should be monitored carefully for evidence of the development of a more severe hepatic reaction while on therapy with CELEBREX. In clinical trials, signs and symptoms consistent with liver disease development or systemic manifestations occurred (e.g., eosinophilia, rash, etc.); CELEBREX should be discontinued.

**Renal Effects:** Long-term administration of NSAIDs has resulted in renal papillary necrosis and other renal injury. Renal toxicity has also been seen in patients in whom prostaglandins have a compensatory role in the maintenance of renal perfusion. In these patients, administration of a nonsteroidal anti-inflammatory drug may cause a dose-dependent reduction in prostaglandin formation and, secondarily, in renal blood flow, which may precipitate overt renal decompensation. Patients at greatest risk of this renal decompensation are those with impaired renal function, heart failure, liver dysfunction, those taking diuretics and ACE inhibitors, and the elderly. Discontinuation of NSAID therapy is usually followed by recovery to the pretreatment state. Clinical trials with CELEBREX have shown renal effects similar to those observed with comparator NSAIDs.

Caution should be used when initiating treatment with CELEBREX in patients with considerable dehydration. It is advisable to rehydrate patients first and then start therapy with CELEBREX. Caution is also recommended in patients with pre-existing kidney disease (see WARNINGS—Advanced Renal Disease).

**Hematological Effects:** Anemia is sometimes seen in patients receiving CELEBREX. In controlled clinical trials the incidence of anemia was 0.6% with CELEBREX and 0.4% with placebo. Patients on long-term treatment with CELEBREX should have their hemoglobin or hematocrit checked if they exhibit any signs or symptoms of anemia or blood loss. CELEBREX does not generally affect platelet counts, prothrombin time (PT), or partial thromboplastin time (PTT), and does not appear to inhibit platelet aggregation at indicated dosages (See CLINICAL STUDIES—Special Studies—Platelets).

**Fluid Retention and Edema:** Fluid retention and edema have been observed in some patients taking CELEBREX (see ADVERSE REACTIONS). Therefore, CELEBREX should be used with caution in patients with fluid retention, hypertension, or heart failure.

**Preexisting Asthma:** Patients with asthma may have aspirin-sensitive asthma. The use of aspirin in patients with aspirin-sensitive asthma has been associated with severe bronchospasm which can be fatal. Since cross reactivity, including bronchospasm, between aspirin and other nonsteroidal anti-inflammatory drugs has been reported in such roid anti-inflammatory drugs has been reported in such aspirin-sensitive patients, CELEBREX should not be administered to patients with this form of aspirin sensitivity and should be used with caution in patients with preexisting asthma.

**Information for Patients:** CELEBREX can cause discomfort and, rarely, more serious side effects, such as gastrointestinal bleeding, which may result in hospitalization and even fatal outcomes. Although serious GI tract ulcerations and bleeding can occur without warning symptoms, patients should be alert for the signs and symptoms of ulcerations and bleeding, and should ask for medical advice when observing any indicative signs or symptoms. Patients should be apprised of the importance of this follow-up (see WARNINGS—Risk of Gastrointestinal Ulceration, Bleeding and Perforation).

Patients should promptly report signs or symptoms of gastrointestinal ulceration or bleeding, skin rash, unexplained weight gain, or edema to their physicians.

Patients should be informed of the warning signs and symptoms of hepatotoxicity (e.g., nausea, fatigue, lethargy, pruritus, jaundice, right upper quadrant tenderness, and "flu-like" symptoms). If these occur, patients should be instructed to stop therapy and seek immediate medical therapy.

Patients should also be instructed to seek immediate emergency help in the case of an anaphylactoid reaction (see WARNINGS).

In late pregnancy CELEBREX should be avoided because it may cause premature closure of the ductus arteriosus.

Patients with familial adenomatous polyposis (FAP) should be informed that CELEBREX has not been shown to reduce the colorectal, duodenal or other FAP-related cancers, or the need for endoscopic surveillance, prophylactic or other FAP-related surgery. Therefore, all patients with FAP should be instructed to continue their usual care while receiving CELEBREX.

**Laboratory Tests:** Because serious GI tract ulcerations and bleeding can occur without warning symptoms, physicians should monitor for signs or symptoms of GI bleeding.

During the controlled clinical trials, there was an increased incidence of hyperchloremia in patients receiving celecoxib compared with patients on placebo. Other laboratory abnormalities that occurred more frequently in the patients receiving celecoxib included hypophosphatemia, and elevated BUN. These laboratory abnormalities were also seen in patients who received comparator NSAIDs in these studies. The clinical significance of these abnormalities has not been established.

**Drug Interactions**

**General:** Celecoxib metabolism is predominantly mediated via cytochrome P450 2C9 in the liver. Co-administration of celecoxib with drugs that are known to inhibit 2C9 should be done with caution.

*In vitro* studies indicate that celecoxib, although not a substrate, is an inhibitor of cytochrome P450 2D6. Therefore, there is a potential for an *in vivo* drug interaction with drugs that are metabolized by P450 2D6.

**ACE-inhibitors:** Reports suggest that NSAIDs may diminish the antihypertensive effect of Angiotensin Converting Enzyme (ACE) inhibitors. This interaction should be given

consideration with ACE-inhibitors. **Furosemide:** Observations, triuretic effect. This response prostaglandin. **Aspirin:** CE However, CELEBREX ation or oth BREX alone. Gastrointes CELEBREX lar prophyls **Fluconazole** 200 mg QC plasma cont of celecoxib Pharmacoki troduced at ing flucona: **Lithium:** I steady-stat 17% in sub BREX 200 ium alone. monitored **Methotrex** thritis pat have a sign trexate.

**Warfarin:** ticularly in CELEBREX ilar agent: bleeding c coagulant healthy st rin. In the lant effect However, have beer ation with ing CELI Carcinog Celecoxib 200 mg/l mately 2 AUC<sub>0-24</sub> mg/kg fo equal to 200 mg Celecoxib tion assi togenic i an *in vi* Celecoxib oral dos man exj **Pregna** **Teratog** not ters (equal t AUC<sub>0-24</sub> mately by AUC such a shaper phragr at oral exposu no stu during potent **Nonte** tion a surviv matel mg B prost altere pecte ducte the d BRE: avoid Labo layer rats the / labori Nurs tatir not Becu caus ing whe tak mot Ped Saf of 1